Novel strategies in dendritic-cell based immunotherapy

Focusing on adjuvant treatment of stage III melanoma

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The research presented in this thesis was performed at the Department of Medical Oncology and Department of Tumor Immunology of the Radboud university medical center and was established by collaboration with the Departments of Surgery, Hematology and Dermatology of the Radboud university medical center.

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General introduction and outline of the thesis

Adapted from Frontiers in Immunology, 2018.

General introduction

Immunotherapy of cancer

Since William Coley made his early contributions to the study of cancer immunotherapy in the 1890s, harnessing the capabilities of the immune system to eliminate cancer cells remained a long-sought dream.[1] Occasional reports of spontaneously regressed melanoma, regarded as an immune-mediated phenomenon, led researchers to consider melanoma as an immunogenic tumor.[2] This triggered the focus of research on melanoma to realize this dream of combating cancer with immune-modulating strategies. In the last decade, after more than hundred years of research, efforts were finally rewarded with the introduction of immune checkpoint inhibitors (ICI). ICI showed that immunotherapy is able to induce important clinical responses which revolutionized the treatment of cancer. The importance of this finding is endorsed by the awarding of the researchers that founded this new form of anti-cancer treatment, James P. Allison and Tasuku Honjo, with the 2018 Nobel Prize in Physiology or Medicine. The success of ICI spurred a considerable amount of research activity into the field of immunotherapy. Immunotherapy encompasses more than ICI alone. Dendritic cell (DC) vaccination is an alternative form of immunotherapy and a prime candidate to enrich the treatment possibilities for cancer. Considering the fact that the field of immunotherapy is moving fast, it is of utmost importance to delineate the position of DC vaccines in the therapeutic landscape of melanoma and other malignancies.

Melanoma

Melanoma is a form of skin cancer which arises from melanocytes and can behave aggressively due to its potential to metastasize. Since 1972 the chemotherapeutic agent dacarbazine was considered standard of care in the treatment of metastatic melanoma. The minor response rate around 13% led to a poor prognosis at that time, with a median overall survival (OS) between 6 and 11 months.[3] Several other regimens incorporating dacarbazine have been tested, unfortunately without improving survival.[3] Due to this poor prognosis, it is important to prevent metastatic melanoma by surgical resection at an early stage of the disease. Risk of recurrent disease impacts prognosis, in particular when the regional lymph nodes are involved (stage III melanoma) and despite complete resection. This risk depends on the tumor characteristics dividing stage III disease in stages IIIA, IIIB and IIIC with decreasing 5-year OS.[4] Adjuvant treatment decreasing the rate of recurrent disease and increasing survival is therefore warranted and an important focus of this thesis.

Evolving therapeutic landscape in metastatic melanoma

The last decade, major advantages significantly improving the prospects for metastatic and unresectable stage III melanoma patients, have been made. These consist of the approval of targeted therapy with BRAF and MEK inhibitors and immunotherapy with ICI and an oncolytic

virus therapy. Targeted therapy with BRAF and MEK inhibitors is registered for treatment of patients with a melanoma harboring an activating mutation in the BRAF gene. The use of BRAF inhibitors led to a response rate of 48% compared to 5% in the dacarbazine group. Median progression-free survival was 5.3 months compared to 1.6 months in the dacarbazine group (hazard ratio (HR) 0.26, $p < 0.001$).[5] In 2011, these results led to the approval of the first BRAF inhibitor in metastatic melanoma. Acquired resistance is an important reason of treatment failure with BRAF inhibition and is mostly the result of a downstream reactivation of the mitogen-activated protein kinase (MAPK) pathway. MEK inhibitors were developed to overcome this resistance. In 2014, the first combined treatment with a BRAF and MEK inhibitor was approved for metastatic disease, as it lifted the progression-free survival from 7.2 months with BRAF monotherapy to 12.3 months in BRAF and MEK combination therapy (HR 0.70, $p = 0.005$).[6]

The clinical application of approved immunotherapy is currently defined by ICI and talimogene laherparepvec (T-VEC), an oncolytic virus therapy. The latter was added to the approved therapies in 2015 and consists of intralesional application of the virus in patients with unresectable cutaneous, subcutaneous and nodal melanoma metastases. T-VEC is a herpes simplex virus genetically modified to only replicate in and lyse tumor cells and the GM-CSF gene is transfected for the local production of GM-CSF by the tumor cells leading to attraction of DCs. It improves durable response rates compared to GM-CSF alone.[7] The other immunotherapeutic agents, ICI, are monoclonal antibodies (mAbs) that target immune checkpoint molecules such as cytotoxic T-lymphocyte-associated antigen (CTLA)-4, programmed death (PD)-1 and programmed death ligand (PD-L)1. These molecules have immune response inhibiting functions and are involved in the prevention of autoimmunity and the maintenance of peripheral tolerance. It is well known that tumor cells are able to upregulate the expression of inhibitory checkpoint molecules, leading to inhibition of the immune response. CTLA-4, PD-1 and PD-L1 have distinct functions. CTLA-4 exerts its inhibitory functions on the initial T-cell activation whereas PD-1 and PD-L1 mainly have roles in the inhibition of the effector functions of T cells.[8, 9] ICI antagonize these checkpoint molecules and thereby aim to augment the anti-cancer immune response. In 2010, ipilimumab (mAb targeting CTLA-4) was the first immunotherapeutic agent providing clinical benefit in cancer patients, extending median OS of metastatic melanoma patients to 10 months, compared to 6.4 months for the control group receiving a gp100 peptide vaccine.[10] With an overall response rate (ORR) of 11-19%, ipilimumab was a great improvement compared to the standard of care at the time, but it still offered clinical benefit in only a minority of melanoma patients.[11, 12] However, a major advantage is the durable clinical benefit in a substantial portion of responding patients.[13] In 2014, two mAbs targeting the PD-1 molecule (nivolumab and pembrolizumab) were also approved for the treatment of metastatic melanoma. Compared to ipilimumab, PD-1 inhibition achieves a higher ORR of 33-44%.[14-16] After the landmark studies in melanoma, research into ICI accelerated with the exploration of ICI in other cancer types and use of different checkpoint molecules of which the PD-L1 targeting mAbs avelumab, atezolizumab and durvalumab are

at present approved in a number of malignancies other than melanoma.[17] With the addition of the PD-1 targeting agent cemiplimab for cutaneous squamous cell carcinoma, the field of ICI now encompasses 7 Food and Drug Administration (FDA) and European Medicines Agency (EMA) approved mAbs (**Table 1**)**.** The number of indications is likely to grow as ICI are currently tested in a large number of additional malignancies.[18]

Table 1 Indications of the seven currently approved monoclonal antibodies in the treatment of cancer (as of April 2019)

Monoclonal antibody	Target	FDA/EMA-approved indications
Ipilimumab	CTLA-4	**Melanoma**
Pembrolizumab	PD-1	Cervical cancer, gastric/ gastroesophageal cancer, HCC, HNSCC, Hodgkin's lymphoma, MCC, **melanoma**, MSI-high cancer, NSCLC, PMBCL, UCC
Nivolumab	PD-1	HCC, HNSCC, Hodgkin's lymphoma, **melanoma**, MSI-high/dMMR CRC, NSCLC, RCC, UCC
Atezolizumab	PD-L1	NSCLC, TNBC, UCC
Avelumab	PD-L1	MCC, UCC
Durvalumab	PD-L1	NSCLC, UCC
Cemiplimab	PD-1	CSCC
Combined treatment with ipilimumab and nivolumab	CTLA-4/PD-1	**Melanoma**, MSI-high/dMMR CRC, RCC

CRC colorectal cancer, *CSSC* cutaneous squamous cell carcinoma, *CTLA-4* cytotoxic T-lymphocyte-associated protein 4, *dMMR* DNA mismatch repair deficiency, *EMA* European Medicines Agency, *FDA* Food and Drugs Administration, *HCC* hepatocellular carcinoma, *HNSCC* head and neck squamous cell carcinoma, *MCC* merkel cell carcinoma, *MSI* microsatellite instability, *NSCLC* non-small-cell lung carcinoma, *PD-1* programmed cell death protein, *PD-L1* programmed death-ligand 1, *PMBCL* primary mediastinal large B-cell lymphoma, *RCC* renal cell carcinoma, *TNBC* triple-negative breast cancer, *UCC* urothelial cell carcinoma

ICI come with a different toxicity profile compared to other types of anti-cancer therapeutics, caused by specific immune-related side effects. In metastastic melanoma, monotherapy with anti-PD-1 and anti-CTLA-4 mAbs is associated with around 12-13% and 20-27% treatment-related grade 3-5 adverse events, respectively.[10-12] For ICI as adjuvant therapy these rates are slightly higher for anti-PD-1 mAbs with 14-15% treatment-related grade 3-5 adverse events, but for ipilimumab these rates rise to 46% in the adjuvant setting with the dosage of 10mg/kg, compared to 3mg/kg used in the metastastic setting.[19, 20] Especially in adjuvant treatment with high life expectancy when melanoma is cured, favorable toxicity profiles are of great importance to maintain quality of life, warranting therapies with a low number of severe (long-term) adverse events.

Dendritic cell vaccination

Since their discovery by Ralph M. Steinman and Zanvil Cohn in 1973, it became clear that DCs are antigen-presenting cells crucial in activating the adaptive immune response.[21] DCs are spread throughout the body, constantly monitoring their surroundings for antigens and danger signals. Once stimulated by an activating stimulus, DCs undergo maturation and migrate to lymphoid organs where they activate effector cells of the immune system, primarily T and B cells.[22] Through this process, DCs are vital for immunosurveillance. Immunosurveillance signifies the crucial role of the immune system in the detection and elimination of both pathogens and cancer cells. However, immunosurveillance occasionally fails as tumors sometimes silence an initiated immune response or fail to express the "danger signals" necessary for the activation of the immune system. When the process of immunosurveillance fails, one of the hurdles for the outgrowth of cancer cells is omitted. DC vaccination aims to correct this failure by reversing the ignorance of the immune system to malignant cells. To achieve this, DCs are stimulated *ex vivo* with danger signals and loaded with tumor antigen(s) presented on their major histocompatibility complex (MHC) molecules. Subsequently, mature tumor antigen-loaded DCs are injected into the patient to activate antigen-specific T cells which selectively eliminate tumor cells expressing the tumor antigen (**Figure 1**). The majority of research groups, including our own, employ treatment schemes with multiple administrations of DCs to induce immunological memory.[23]

DC vaccines are produced following some basic principles (**Figure 2**). Naturally circulating DCs or monocytes are isolated from autologous peripheral blood mononuclear cells obtained by apheresis. In case of monocytes, *ex vivo* differentiation into DCs is required. Both naturally circulating DCs and monocyte-derived DCs are matured as this is essential for effective T cell activation.[24-26] Maturation is associated with functional and morphological changes in DCs. Following maturation, DCs enhance their expression of MHC I and II and co-stimulatory molecules and have an increased capability of cytokine production. These processes are vital, as not or incompletely matured DCs can induce tolerance rather than immunity.[27] During vaccine manufacturing, DCs are loaded with relevant tumor antigen(s) to induce a tumor-specific immune response *in vivo*. After passing quality control, vaccines are administered to the patient.

After the first clinical trial report in 1996[28], multiple small clinical trials have been conducted with different forms of DC vaccination. In 2006, a phase III trial investigating DC vaccination in metastatic melanoma patients was stopped prematurely due to lack of efficacy compared to dacarbazine.[29] However, thereafter multiple potential improvements were exploited. Despite the basic principles as aforementioned, the specific details of DC vaccination manufacturing in trials vary widely. Differences in these protocols cover all aspects of DC vaccination including culture methods, the usage of DC subsets, maturation

methods, antigen loading techniques, used antigens and the route of administration. Especially, the subset of DC used, the method of maturation and the choice of antigen(s) are subject of intense research. For example, several groups, including our own, currently use naturally circulating DCs instead of monocyte-derived DCs. Naturally circulating DCs do not require extensive culturing which is believed to retain their functionality. Different maturation techniques are also being explored, such as the use of toll-like receptor ligands or electroporation with mRNA encoding proteins that induce DC maturation.[30, 31] Several methods to load DCs with antigens exist and were tested in different trials, but little trials directly compare them.[32] The goal of DC vaccination is to kill tumor cells by the induction of functional antigen-specific T cells *in vivo*.[33] Despite challenges associated with measuring the immunological effect of DC vaccination, immunological endpoints are reported in a substantial portion of phase I/II clinical DC vaccination trials. Several studies report the generation of antigen-specific T cells to be positively correlated with survival, strengthening the believe that DC vaccination can result in clinical benefit.[34-36]

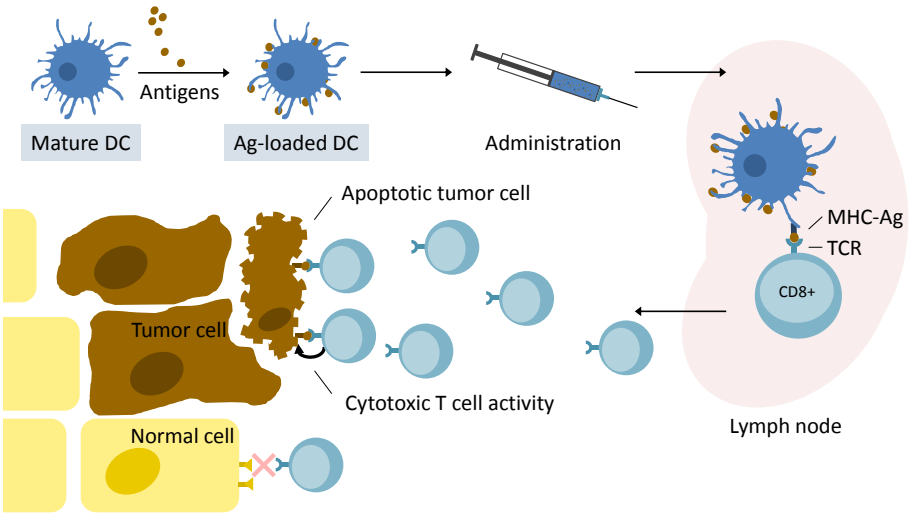

Figure 1 Induction of a tumor-specific immune response by dendritic cell vaccination
Dendritic cells, loaded *ex vivo* with tumor antigen(s), activate tumor antigen-specific T cells after administration to the patient. Activated T cells subsequently patrol the body in search of their respective antigen. When their target is found, T cells exert their cytotoxic functions on tumor cells. Antigen-specific triggering of T cells refrains T cells from non-specific killing and off-target toxicity. *Ag* antigen, *CD8* cluster of differentiation 8 (cytotoxic T cell), *DC* dendritic cell, *MHC* major histocompatibility complex, *TCR* T cell receptor

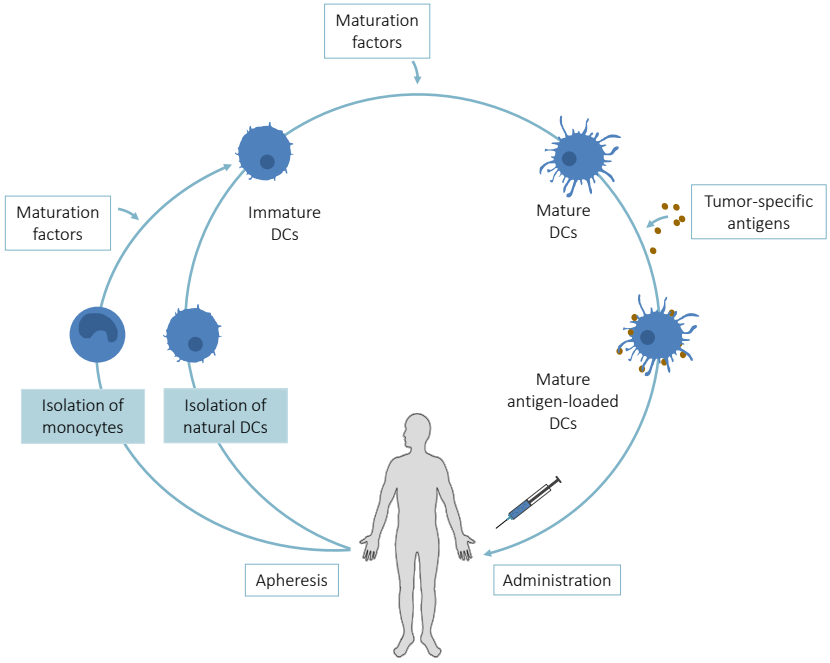

Figure 2 Process of generating dendritic cell vaccines

Autologous dendritic cells or monocytes are obtained via an apheresis procedure. Monocytes first have to be differentiated into dendritic cells. Subsequently, dendritic cells are matured *ex vivo* and loaded with tumor antigen. Finally, the dendritic cells are administrated to the patient.

Regardless of the precise protocol employed, DC vaccination is associated with a very favorable toxicity profile. The majority of side effects reported in various clinical trials were short-lived grade 1 or 2 adverse events, consisting of self-limiting flu like symptoms, fever and local injection site reactions. Treatment-related grade 3 or 4 adverse events following DC vaccination as stand-alone therapy are uncommon.[33, 37]

Tumor-induced immune suppression as a hurdle for clinical response to DC vaccination

For metastatic melanoma survival benefit with ICI is well established and favorable when compared to DC vaccination in terms of response rate: ORR 11-19% on anti-CTLA-4 mAb, 33-44% on anti-PD-1 mAb, and 58% when combined with anti-CTLA-4 mAb.[10-12, 14] ORR after treatment with DC vaccination is approximately 8.5% in metastatic melanoma patients.[37] Due to its less established clinical benefit, it is unlikely for DC vaccination to gain a role as monotherapy in widespread metastatic disease. Despite limited clinical response, the generation of a cellular immune response upon DC vaccination is commonly reported.[37] This contrasting finding might be due to immune suppressive factors in the TME leading to anergy of effector T cells induced by DC vaccination. With advancing knowledge of the interaction between the immune

system and cancer, it becomes increasingly clear that higher tumor load is associated with higher tumor-induced immune suppression (**Figure 3**). For example, regulatory T cells (Tregs) and myeloid derived suppressor cells (MDSCs) induce anergy in T cells.[38] Moreover, several soluble factors secreted by tumor cells, such as transforming growth factor (TGF)-β, interleukin (IL)-10 and vascular endothelial growth factor (VEGF), are recognized to suppress infiltrated effector T cells.[39-41] Also, tumors are able to upregulate indoleamine 2,3-dioxygenase (IDO)1 which converts tryptophan to kynurenine, inhibiting effector T cells through a mechanism not completely understood.[42]

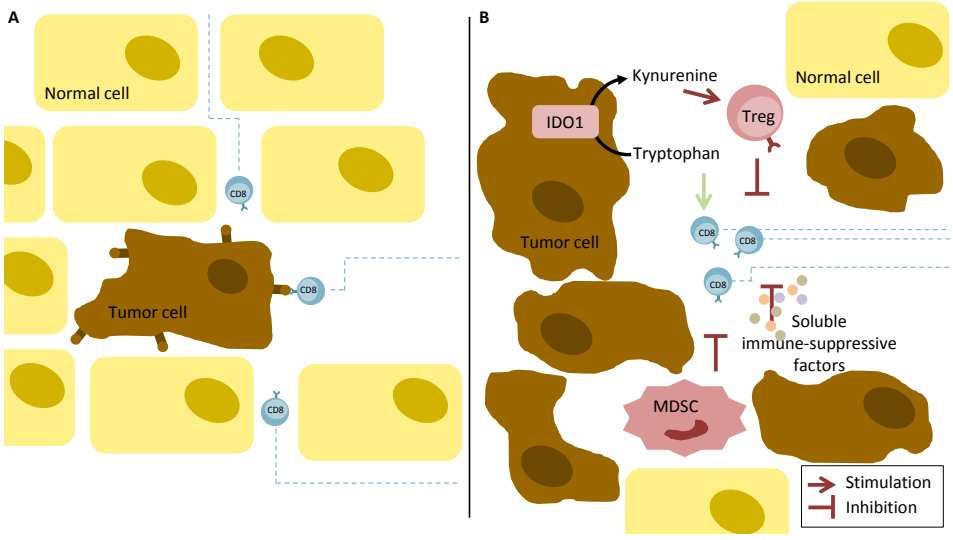

Figure 3 The difference in tumor load-associated immune suppression between minimal residual disease and a situation with high tumor load

Antigen-specific T cells induced by dendritic cell vaccination. (**A**) Minimal residual disease (e.g. after surgical resection with curative intent) is associated with less immune suppression as opposed to (**B**) a situation with a higher tumor load. Tumor-associated immune suppression is caused by (among other factors) Tregs, MDSCs, soluble immune suppressive factors (such as IL-10, TGFβ and VEGF) and IDO1 activity. Vaccination-induced effector T cells can be rendered anergic by this immune suppression, resulting in lower clinical response rates. Therefore, dendritic cell vaccination might be more effective in the adjuvant setting. *$CD8^+$* (cytotoxic) T cell, *IDO1* indoleamine 2,3-dioxygenase 1, *MDSC* myeloid derived suppressor cell, *Treg* regulatory T cell

A strategy to overcome this immune-suppressive hurdle for DC vaccination to induce clinical response, is combination with therapies modulating these immune-suppressive factors in the TME. For example, chemotherapy, radiation therapy or targeted therapy can alleviate tumor burden and thereby tumor-induced immune suppression. In addition, although these modalities are not classically regarded as immunotherapeutics, they also possess immune-modulating effects.

Chemotherapeutics are associated with lymphodepletion, but positive immune-modulatory effects are described and include the induction of immunogenic cell death and depletion of Tregs and MDSCs by platinum compounds.[43, 44] Combined therapy of DC vaccination and such immune-modulating chemotherapeutic agents may therefore be an interesting approach. Besides combining DC vaccination with therapies combating the immune-suppressive TME, DC vaccination can also be applied in the adjuvant setting where immune suppression by the TME is naturally lower. Surgical resection with curative intent aims to excise all tumor burden. However, occult residual disease remains in a variable portion of patients and can eventually lead to relapse.[45] Adjuvant treatment aims at killing residual cancer cells, thereby reducing the rate of relapse. In the adjuvant setting, the possibly remaining occult disease represents a low tumor burden, hence less immune suppression. As tumor-induced immunosuppression might be an underlying cause of the low clinical response to DC vaccination in metastatic disease, DC vaccination may be more successful in the adjuvant compared to the metastatic setting. Indeed, in our group we detected antigen-specific T cells in 71% of stage III melanoma patients following adjuvant DC vaccination compared to 23% following vaccination in the metastatic setting.[46] Besides, the OS of stage III melanoma patients receiving adjuvant monocyte-derived DCs was improved compared to a matched control cohort.[47]

Emerging adjuvant treatment options in stage III melanoma

Despite surgical resection, melanoma has a risk of relapse which is particularly high when the disease has metastasized to regional lymph nodes. Prior to the current therapeutic era containing ICI and targeted therapy, stage III melanoma had 5-year OS rates ranging from 40% (stage IIIC) to 78% (stage IIIA).[4] These rates implicate a very high risk of recurrent disease for these patients, despite complete surgical resection. Therefore, adjuvant therapy for this group of patients is warranted. Melanoma is very chemoresistant and the adjuvant treatment strategy in the pre-ICI and targeted therapy era was mainly based on cytokine treatment with interferon-α and radiotherapy. However, interferon-α provided little survival benefit and is associated with significant toxicity.[48] Adjuvant radiotherapy showed an improved recurrence-free survival (RFS), without benefit in OS.[49]

Results of recent phase III trials changed the immunotherapeutic options for these patients. Ipilimumab showed clinical activity in the adjuvant setting for stage IIIA, IIIB and IIIC melanoma with a 5-year RFS rate of 40.8% compared to 30.3% in the placebo group (HR for recurrence or death, 0.76; $p < 0.001$).[50] Importantly, 5-year OS rate was also improved to 65.4% compared to 54.4% (HR for death 0.72; $p = 0.001$).[51] Based on these data, ipilimumab was approved as adjuvant therapy by the FDA in 2015. However, the application of adjuvant ipilimumab is opposed by its significant toxicity: 42-46% of patients experienced treatment-related grade 3-4 adverse events and ≤ 1% patients died due to adverse events.[19, 51] In addition to ipilimumab, both nivolumab and pembrolizumab have shown to increase RFS in the adjuvant setting.[19, 20] Adjuvant nivolumab was tested against ipilimumab in completely resected stage IIIB, IIIC and IV

melanoma. In this study nivolumab improved the 1-year RFS rate of stage IIIB and IIIC patients to 72.3% compared to 61.6% in ipilimumab-treated patients (HR for recurrence or death 0.65, $p < 0.001$).[19] Adjuvant pembrolizumab was compared to placebo in stage IIIA, IIIB and IIIC melanoma. The 1-year RFS rates were 75.4% and 61.0%, respectively (HR for recurrence or death 0.74, $p < 0.001$).[20] Toxicity of anti-PD1 mAbs is lower compared to ipilimumab, as it results in treatment-related grade 3-5 adverse events in 14-15% of patients.[19, 20] Both the FDA and EMA granted approval for adjuvant nivolumab and pembrolizumab while data on OS are pending. In addition to ICI, targeted therapy with combined dabrafenib and trametinib (BRAF and MEK inhibitor) is approved as adjuvant therapy for BRAF-mutated stage IIIA, IIIB and IIIC melanoma. This combination showed an OS benefit at 3 years of 86% compared to 77% in the placebo group (HR for death 0.57, $p = 0.0006$). Of the patients treated with combined dabrafenib and trametinib, 41% experienced grade 3 or 4 adverse events.[52]

In stage III melanoma, DC vaccination has also been investigated as adjuvant treatment. Retrospective analysis from our group showed clinical benefit in stage III melanoma patients adjuvantly treated with monocyte-derived DC vaccination compared to matched controls. In this study, OS of 78 patients treated with DC vaccines doubled compared to the OS of 209 controls (63.6 months vs. 31.0 months; HR 0.59; $p = 0.018$).[47] Markowicz et al. have shown similar results in a prospective study with a peptide-loaded DC vaccine. In 22 vaccinated patients the study achieved a 3-year OS of 68% compared to 26% in the 22 patients of the matched historical control group ($p = 0.029$). The primary endpoint however, 3-year RFS rate, was not significantly improved, possibly due to the small number of patients (vaccinated patients: 41%; controls 15%; $p = 0.108$).[53] Currently, no phase III trials have been completed with adjuvant DC vaccination of melanoma. To date, too little data is available to claim that DC vaccination is effective in the adjuvant setting. Yet, the above presented data show favorable clinical results and confirm the limited toxicity which might also result in a better health-related quality of life during and after therapy. Especially in the adjuvant setting, where part of patients will not endure a relapse even without adjuvant therapy, it is important to take the toxicity of the therapy into account. In addition, an important portion of melanoma patients is young and of working-age, making toxicity and its impact on the quality of life even more important. Whether DC vaccination acquires a definite role in the adjuvant treatment of cancer will depend on clinical results of trials investigating adjuvant DC vaccination and results of other ongoing phase III trials assessing other adjuvant treatments. Especially the pending OS data of trials with anti-PD1 mAbs are awaited.

Conclusion

Immunotherapy for the treatment of melanoma is a fast-moving field. It is important to determine the relative position of DC vaccination to other therapies in this rapidly evolving landscape. With the proven OS benefit of ICI and the lesser clinical benefit of DC vaccination in metastatic disease, it becomes increasingly clear that the future of DC vaccination in extensive metastatic

disease as standalone treatment is probably limited. The immune suppression that needs to be overcome for DC vaccination to induce higher clinical response rates is an important aspect for future research. This could, for example, be dealt with by combination of DC vaccination with immune-modulating agents such as platinum compounds. In addition, the lower tumor burden, hence lesser immune suppression, in stage III melanoma and the favorable toxicity profile of DC vaccination, make this therapy an ideal candidate to be explored as adjuvant treatment option. Another important focus is optimizing the DCs used in vaccination, for example with use of naturally occurring DCs.

Consequently, for DC vaccination to gain a definitive role in the therapeutic landscape of cancer, research should be focused on well-designed trials in which optimization of the DCs used in vaccines, application in the adjuvant setting and combinational strategies are explored, taking toxicity and quality of life into account.

Outline of the thesis

The overall aim of the thesis is to explore different strategies to optimize DC vaccination and to provide insight in the position of adjuvant dendritic cell (DC) vaccination in stage III melanoma patients.

Optimizing dendritic cell vaccination

Tumor-induced immune suppression might be a factor limiting the efficacy of immunotherapy, including DC vaccination. We performed a prospective clinical trial in which cisplatin was added to DC vaccination, to counteract the tumor-induced immune suppression. Cisplatin is part of the group of platinum-based chemotherapeutics. Besides its cytotoxicity, cisplatin has immune-modulating properties downregulating immunosuppressive factors in the tumor microenvironment. Therefore, we hypothesized that the addition of cisplatin to DC vaccination might have a synergistic effect. In **chapter 2** the results of our prospective clinical trial investigating combination therapy with DC vaccination and cisplatin are described. In the trial, the main focus was feasibility and safety, but also differences in immunological and clinical responses are studied. Besides improving the efficacy of DC vaccination by combination treatment, we searched for methods to improve the potency of DCs used in the vaccine. After years of trials with monocyte-derived DCs, a strategy was developed to directly select the scarce naturally circulating DCs from the blood. Due to the omission of the long culture period needed to differentiate DCs from monocytes *ex vivo*, potentially hampering their functionality, the use of naturally occurring DCs might lead to improved treatment outcome. After confirming the feasibility and safety of this novel approach in metastatic melanoma patients, we performed a clinical trial in stage III melanoma patients comparing different naturally circulating DC subsets, myeloid and plasmacytoid DCs, and the combination of both subsets. In **chapter 3** the feasibility and safety of the combined vaccination with myeloid and plasmacytoid DCs and the immunological and clinical response in patients vaccinated with the two subsets alone or combined are described.

Important aspects of adjuvant immunotherapy in stage III melanoma

An important portion of patients with stage III melanoma will not encounter a relapse, even without adjuvant therapy. However, in this evolving field of adjuvant treatment, patients are exposed to possible (long-term) side effects. As the majority of melanoma patients is young and of working-age, toxicity of adjuvant therapy and its impact on the health-related quality of life is even more relevant. Therefore, we prospectively evaluated the health-related quality of life of stage III melanoma patients adjuvantly treated with DC vaccination, which is described in **chapter 4**. Adjuvant treatment of stage III melanoma patients starts shortly after complete surgery. Prior to the surgical removal of metastases, stage IIIB and IIIC melanoma patients undergo imaging to exclude distant metastases. The aim of adjuvant therapy is to eliminate

undetectable micrometastases to reduce the rate of recurrent disease. **Chapter 5** addresses the rate of relapse, that occurs as early as prior to the start of adjuvant therapy and despite prior exclusion of distant metastases and after complete resection of the lymph node metastases. The rate of these early relapses is relevant for patients planned to start adjuvant treatment with DC vaccination or the currently approved adjuvant treatment options with immune checkpoint inhibitors and targeted therapy. In **chapter 6,** the findings of this thesis are summarized and future perspectives are discussed**.**

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2

Autologous monocyte-derived DC vaccination combined with cisplatin in stage III and IV melanoma patients: a prospective, randomized phase 2 trial

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Abstract

Background. Autologous monocyte-derived dendritic cell (DC) vaccines can induce tumor-specific T cells, but these immune responses can be counteracted by several immunosuppressive mechanisms. Cisplatin has shown immunomodulatory effects *in vivo* and may therefore facilitate induction of T cell responses, hence have an additive effect on DC vaccination.

Methods. Prospective, randomized, open label phase 2 study (NCT0228541) including stage III and IV melanoma patients receiving 3 biweekly vaccinations of gp100 and tyrosinase mRNA-loaded monocyte-derived DC with or without cisplatin. Primary objectives were immunogenicity and feasibility, and secondary objectives were toxicity and survival.

Results. Twenty-two stage III and 32 stage IV melanoma patients were included in the analysis. DC vaccination induced tumor-specific tetramer-positive $CD8^+$ T cells in 44% versus 67% of patients and functional T cell responses in 28% versus 19% of skin-test infiltrating lymphocytes in patients receiving DC vaccination with cisplatin versus DC vaccination monotherapy, respectively. Four patients stopped cisplatin treatment because of adverse events and continued DC vaccination monotherapy. No therapy-related grade 3 or 4 adverse events occurred in the DC monotherapy group. During combination therapy with cisplatin one therapy-related grade 3 adverse event, decompensated heart failure due to fluid overload, occurred. In stage III melanoma patients, median recurrence-free survival was 45.9 months after combination treatment compared to 9.6 months in the group treated with DC monotherapy ($p = 0.245$). In stage IV patients, median progression-free survival was 4.7 months after combination treatment versus 3.0 months after DC monotherapy ($p = 0.101$).

Conclusions. Combination of DC vaccination and cisplatin in stage III and IV melanoma patients is feasible and safe, but does not seem to result in more tumor-specific T cell responses or improved clinical outcome, when compared to DC vaccination monotherapy.

Introduction

Based on their capacity to activate and prime naïve T cells, dendritic cells (DCs) are the most efficient antigen-presenting cells of the immune system. This makes them ideal candidates to be exploited for vaccination therapy.[1] Studies with autologous monocyte-derived DC vaccines have shown to induce tumor-specific immune responses in both lymph-node involved (stage III) and metastastic (stage IV) melanoma patients, but with higher immunological response rates in stage III patients.[2] Despite immunological responses, objective clinical responses are rare in stage IV melanoma patients treated with DC vaccination alone.[3-5] Compared to stage IV disease, a higher percentage of stage III patients with immunological response to DC vaccination could be explained by a lower tumor burden and concomitant less immune suppression. Accordingly, we found favorable overall survival (OS) in stage III melanoma patients who received adjuvant DC vaccination compared to matched controls.[6] These results have to be confirmed in our randomized phase 3 trial (NCT02993315).

Combination with other therapies might improve the efficacy of DC vaccination. In particular, therapies modulating an immunosuppressive tumor microenvironment (TME) may strengthen the effect of DC vaccination. Platinum-based compounds, a group of chemotherapeutic agents, are widely used for several forms of cancer.[7, 8] The oldest platinum compound, cisplatin, was also tested in metastatic melanoma patients, as monotherapy and in combination with other types of chemotherapy, interferon (IFN) or interleukin (IL)-2. However without great clinical benefit and with more toxicity than the commonly used chemotherapeutic for melanoma, dacarbazine. Therefore, the latter was the preferred systemic therapy at time of trial enrollment, this was before the approval of immune checkpoint inhibitors (ICI).[9-12] The rationale behind the combination of DC vaccination with cisplatin is based on the ability of cisplatin to not only cross-link DNA and inhibit mitosis, but to also have immunomodulatory effects.[13] *In vitro* platinum drugs cause inhibition of signal transducer and activator of transcription (STAT) via decreasing phosphorylation of different STAT proteins.[14] STAT proteins each have a different effect on the antitumor response.[15, 16] For example, diminished STAT6 phosphorylation results in downregulation of T cell-inhibitory molecule programmed death ligand 2 (PD-L2) on both DCs and tumor cells. PD-L2 downregulation results in enhanced tumor cell recognition by T cells.[8, 16] More recently, preclinical studies showed that cisplatin may improve the ability of cytotoxic T cells to recognize cancer cells by upregulating MHC class I expression on tumor cells and may cause upregulation of the lytic activity of cytotoxic effector cells.[13] Furthermore, it has been shown that cisplatin may improve recruitment of immune effector cells to the TME and enhances proliferation of these cells and at the same time causes downregulation of immunosuppressive cells in the TME by reducing levels of myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Tregs).[13, 17] Besides this effect in the TME, a reduction

in circulating Tregs was found in patients with non-small cell lung cancer after treatment with cisplatin and vinorelbine.[18] Finally, contrary to previous reports, we recently observed that cisplatin can induce immunogenic cell death.[19] Immunogenic cell death is a mechanism whereby cell death results in an adaptive immune response.

In a preclinical tumor model, synergy between vaccination with synthetic long peptides of human papillomavirus type (HPV) 16 and cisplatin was shown. Combined treatment led to highly infiltrated tumors with HPV-specific tumor necrosis factor (TNF)-α and IFN-γ producing T cells, and it showed significantly decreased tumor cell proliferation compared to single treatment.[20] Combining DC vaccination with cisplatin may have a similar synergistic effect, as the immunomodulatory effects of cisplatin may improve the efficacy of the tumor-associated antigen (TAA)-specific T cells induced by DC vaccination. Thus, the aim of this study was to explore whether the combination of autologous monocyte-derived DC vaccination and cisplatin in stage III and IV melanoma patients is feasible and safe, and whether it leads to better immunological and clinical responses compared to monotherapy with DCs.

Materials and methods

Patient characteristics

Patients between 18 and 70 years of age with histologically confirmed stage III or IV melanoma, both with a cutaneous (American Joint Cancer Committee (AJCC) 7th edition[21]), uveal (AJCC 7th edition[22]) or unknown primary melanoma were eligible for enrollment. Additional key eligibility criteria included: World Health Organization performance status of 0 or 1; melanoma expressing the TAA gp100 (compulsory) and tyrosinase (non-compulsory); normal serum lactate dehydrogenase (LDH); life expectancy of at least 3 months, serum creatinine level of less than 150 µmol/L; start of adjuvant DC vaccination within 2 months of radical lymph node dissection (RLND) in stage III patients; and at least one measurable target lesion according to Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1 in stage IV patients. Key exclusion criteria were: any prior chemotherapy, immunotherapy, or radiotherapy within 4 weeks of the first vaccination; clinical signs or symptoms of brain metastases; rapidly progressive symptomatic disease; a history of any second malignancy in the previous 5 years; serious active infections or autoimmune disease; concomitant use of immunosuppressive drugs; and a known allergy to shell fish due to the use of Keyhole Limpet Hemocyanin (KLH) protein. The trial (NCT0228541) was approved by the Central Committee on Research Involving Human subjects and in concordance with the Declaration of Helsinki. Written informed consent was obtained from all patients.

Study design and treatment

In this prospective, open-label phase 2 study, melanoma patients were randomly assigned in a 1:1 ratio to receive either autologous DC vaccination with cisplatin or DC vaccination alone. Patients were stratified for disease stage (III versus IV). Irresectable stage III patients were considered as having stage IV disease. All patients were vaccinated with autologous cytokine-matured monocyte-derived DC electroporated with mRNA encoding the TAA gp100 and tyrosinase, and pulsed with KLH protein. Patients received 3 biweekly vaccinations, followed by a delayed-type hypersensitivity (DTH) skin test after 1-2 weeks (**Supplementary Figure 1**). In absence of disease progression, patients received 2 additional cycles of vaccinations at 6-month intervals. The vaccinations were administered intradermally and intravenously simultaneously. In the combination arm, 50 mg/m^2 cisplatin intravenously (with a maximum of 100 mg per dose) was administered 1-2 hours before DC injection. The dose of cisplatin was based on *in vitro* experiments, evaluating its effect on cytokine production of DCs.[16] As cisplatin is highly emetogenic, the standard antiemetic regime consisted of dexamethasone (10mg intravenously on day 1 or 12mg orally on day 1-4), aprepitant, ondansetron and metoclopramide. Since February 2014, the dexamethasone regime changed to 12mg orally on day 1-4. Primary objectives of the study were the immunological response and feasibility of the addition of cisplatin to DC vaccination. For

this, patients were considered evaluable when at least one DTH skin test was performed and patients not reaching the primary endpoint were replaced. Secondary objectives were toxicity and survival, consisting of recurrence-free survival (RFS), progression-free survival (PFS), and OS. Toxicity was assessed according to the National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE) version 3.0. Tumor evaluation was performed at baseline and every 3 months thereafter by physical examination in stage III patients and by a CT scan of chest and abdomen evaluated according to RECIST version 1.1 in stage IV patients, until disease recurrence or progression.

Vaccine production

Monocytes were enriched from leukapheresis products by plastic adherence of peripheral blood mononuclear cells (PBMCs) or by counterflow centrifugation using Elutra-cell separator (Gambro BCT, Lakewood, CO) and single-use, functionally sealed disposable Elutra sets, as described before.[23] Monocytes were cultured in X-VIVO 15 medium (Lonza, Walkersville, MD) supplemented with 2% human serum (HS; Sanquin, Amsterdam, the Netherlands) in the presence of IL-4 (500 U/ml), granulocyte macrophage colony-stimulating factor (800 U/ml) (both Cellgenix, Freiburg, Germany) and KLH (10 µg/ml, Calbiochem, Darmstadt, Germany). DCs were matured with a cocktail of 10 ng/ml TNF-α, 5 ng/ml IL-1β, 15 ng/ml IL-6 (all Cellgenix) and prostaglandin E_2 (10 µg/ml, Pharmacia & Upjohn, Puurs, Belgium).[24] Cells used for the DTH skin test were cultured without KLH. DCs were electroporated with mRNA encoding gp100 or tyrosinase, as previously described.[25] Patients could only participate if the produced vaccines met the predefined phenotypic minimal release criteria of mature DCs used in clinical trials.[26] The first vaccine after an apheresis was administered freshly, the remaining vaccines and injections for the DTH skin test were cryopreserved and thawed at the day of administration. DCs were resuspended in NaCl 0.9% (B. Braun, Melsungen, Germany) with 5% HS albumin (Sanquin) and administered both intradermally (maximum of $10x10^6$ cells in 200µl) and intravenously (maximum of $20x10^6$ cells in 1ml). Apheresis was repeated prior to a cycle if necessary to produce a sufficient number of vaccines.

Flow cytometry

Flow cytometry was used to characterize the phenotype of the *ex vivo* generated DCs. The following monoclonal antibodies or appropriate isotype controls were used: anti-HLA-ABC-PE, anti-HLA-DR-PE, anti-CD80-PE, anti-CD83-PE, anti-CD86-PE, anti-CD3-PE, anti-CD25-PE, anti-CD95-PE (all BD Biosciences, San Jose, CA), anti-CD14-PE (Sanquin Reagents, Amsterdam, the Netherlands), anti-HLA-DQ-PE, anti-CD20-PE (both Biolegend, San Diego, CA), and anti-CCR7-PE (Miltenyi Biotec, Bergisch-Gladbach, Germany). For intracellular staining, NKI/beteb (IgG2b; purified antibody) against gp100 and T311 (IgG2a; Cell Marque Corp., Rocklin, CA) against tyrosinase were used. Flow cytometry was done with FACSCalibur flow cytometer equipped with CellQuest software (BD Biosciences).

The presence of Tregs and monocytic (M)-MDSCs was analyzed in PBMCs collected prior to the apheresis and on the day of and prior to the DTH skin test during the first cycle. The antibody panel used for the identification of Tregs consisted of fixable viability dye 780, FoxP3-Alexa488 (both Ebioscience, Vienna, Austria), anti-CD3-BV605 (Biolegend), CD4-BV510 and CD25-BV421 (both BD Biosciences). M-MDSCs were analyzed by staining with CD33-APC (Biolegend), CD14-BV421, HLA-DR-BV510 and CD11b-BV605 (all BD Biosciences) antibodies. Flow cytometry was done using a FACSLyric® equipped with FACSuite software (BD Biosciences). Tregs were identified as $CD3^+CD4^+CD25^+FoxP3^+$ cells. M-MDSCs were analyzed as the percentage of $HLA\text{-}DR^-CD14^+CD11b^+CD33^+$ cells. Analyses were done using FlowJo software version 10.0.7 (Treestar Inc., Ashland, OR).

KLH-specific proliferation

PBMCs were isolated from heparinized blood by Ficoll-Paque density centrifugation and stimulated with KLH (4 µg/2×10^5 PBMC; Immucothel, Biosyn, Carlsbad, CA) in X-VIVO with 2% HS. After 3 days, cells were incubated with ^{3}H-thymidine for 8 hours and incorporation was measured with a β-counter. Experiments were performed in sextuplicate, non-specific proliferation upon stimulation with ovalbumin was used as control. Proliferative response to KLH is given as proliferation index (proliferation with KLH/proliferation without KLH).

Skin-test infiltrating lymphocyte culture analysis

Skin tests were performed between 1 to 2 weeks after each vaccination cycle as described previously.[27] Briefly, DCs electroporated with either gp100, tyrosinase or both antigens (maximum of 1x10^6 DCs in total) were thawed and injected intradermally in the skin of the back of patients at 4 different sites, 4 cm apart from each other. After 2 days, punch biopsies (6 mm) were taken from each of the four injection sites. Half of each biopsy and the other half was manually cut and cultured for 2-4 weeks in RPMI-1640 containing 7% HS and IL-2 (100 U/ml, Novartis, Basel, Switzerland).

Skin-test infiltrating lymphocyte (SKIL) cultures and PBMCs from HLA-A2.1 positive patients were stained with HLA-A2.1 tetrameric-MHC complexes containing the epitopes gp100:154-162, gp100:280-288, or tyrosinase:369-377 (Sanquin) as described before.[28] Tetrameric-MHC complexes recognizing Human Immunodeficiency Virus (HIV) were used as negative control. Tetramer positivity (TM^+) was defined as at least a two-fold increase in the double positive population. In these HLA-A2.1 positive patients, the production of IFN-γ was measured in supernatants after 16 hours of co-culture with different target cells: T2 cells pulsed with gp100:154-162, gp100:280-288, or tyrosinase:369-377; BLM (a melanoma cell line expressing HLA-A2.1 and no endogenous expression of gp100 and tyrosinase) transfected with gp100, tyrosinase or control antigen G250; and Mel 624 (an allogenic HLA-A2.1 positive, gp100-positive, and tyrosinase-positive tumor cell line). Cytokine analysis was performed by cytometric bead array (human Th1/Th2 FlowCytomix multiplex kit, eBioscience), according to

the manufacturer instructions. In HLA-A2.1 negative patients, cytokine production by SKILs was determined by using autologous Epstein-Barr Virus-transformed B (EBV-B) cells as described by van Nuffel and colleagues.[29] Autologous EBV-B cells were generated from PBMCs and electroporated with mRNA encoding full-length gp100 or tyrosinase (Curevac GmbH, Tübingen, Germany). EBV-B cells electroporated with Carcinoembryonic Antigen (CEA)-mRNA were used as a negative control. MRNA-loaded EBV-B cells were co-cultured 1:1 with SKILs for 24 hours and after 24 hours antigen specificity was analyzed by flow cytometry by expression of the early activation markers CD69, CD107a and CD137 on $CD8^+$ T cells. Phorbol myristate acetate (PMA)-stimulated (5 mg/mL; Sigma-Aldrich, Saint Louis, MO) SKILs were used as a positive control. After 24 hours of co-culture, cytokine production was measured with cytometric bead array with the same method as for the HLA-A2.1 positive patients.

Multiplex immunofluorescence staining

Tumor tissue resected prior to the start and after experimental therapy from a comparable organ of origin of the same patient was collected. Formalin-fixed paraffin embedded (FFPE) tumor material of 5 patients (2 stage III and 3 stage IV) in the combination arm and 7 patients (4 stage III and 3 stage IV) in the monotherapy arm was available. From one patient with a partial response to combination therapy, cryopreserved tissue of an excised in transit metastasis prior to the start of therapy was compared to an excised clinically responding in transit metastasis during therapy. The aim was to compare differences in the TME with respect to STAT phosphorylation and T cell infiltrate between patients treated with DC vaccination monotherapy and combination therapy.

Three-color multiplex immunohistochemistry (mIHC) using Opal 7-Color IHC Kit (NEL801001KT, Perkin Elmer, Waltham, MA) was performed manually on 4 µm FFPE or frozen tissue sections for the detection of STAT3 and phosphorylated STAT3 (pSTAT3). Sections from FFPE tissue were first deparaffinized and sections from frozen tissue were first dried, fixed in 4% formaldehyde for 10 minutes and washed in phosphate buffered saline (PBS). Heat-induced epitope retrieval and antibody-TSA complex removals were performed in 10mM citrate buffer (pH 6, CBB999, ScyTek Laboratories, Logan, UT) by microwaving method for 15 minutes after boiling. Tissue sections were subjected to anti-STAT3 1:100 (4904P, clone 79D7, Cell Signaling, Danvers, MA) with Opal 650 and anti-pSTAT3 1:100 (9145L, clone D3A7, Cell Signaling) with Opal 520.

A seven-color mIHC for the detection of different lymphocyte populations was applied using the BOND RX IHC & ISH Research Platform (Leica Biosystems, Wetzlar, Germany) on tissue sections consisting of anti-CD45RO 1:1000 (MS-112, clone UCHL-1; Thermo Scientific, Waltham, MA) with Opal 620, anti-CD8 1:200 (M7103, clone C8/144B; Dako, Santa Clara, CA) with Opal 690, anti-CD20 1:600 (MS-340-S, clone L26; Thermo Scientific) with Opal 570, anti-CD3 1:200 (RM-9107, clone Sp7; Thermo Fisher, Waltham, MA) with Opal 520, anti-Foxp3 1:100 (14-4777, clone 236A/E7; eBioscience Affymetrix) with Opal 540, and a melanoma mix

consisting of HMB-45 1:600 (M063401, clone HMB45; Dako), Mart-1 1:300 (MS-799, clone A103; Thermo Immunologic), Tyrosinase 1:200 (MONX10591, clone T311; Monosan, Uden, the Netherlands), and SOX-10 1:500 (383R, clone EP268; Cell Marque, Rocklin, CA) with Opal 650. All BOND RX epitope retrievals and antibody-TSA complex removals were performed using BOND Epitope Retrieval 2 (AR9640, Leica Biosystems). Blocking steps were performed with Antibody Diluent for 10 minutes, primary antibody incubations for 1 hour, secondary antibody Opal Polymer HRP Ms + Rb incubations for 30 minutes, and Opal reagent incubations for 10 minutes, all at room temperature, either manual or automated. Tissue sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) and mounted with Fluoromount-G (0100-01; Southern Biotech, Birmingham, AL). A similar seven-color mIHC was performed manually on cryo tissue sections with anti-granzyme B 1:300 (ab134933, clone EPR8260; Abcam, Cambridge, United Kingdom) with Opal 690 and anti-CD8 1:1600 with Opal 570, replacing anti-CD20. HIER and antibody-TSA complex removals were performed citrate buffer for only 5 minutes after boiling. For analysis, $CD3^{+}CD8^{-}$ cells were considered $CD4^{+}$ T cells.

The slides were scanned using the Automated Quantitative Pathology Imaging System Vectra 3.0.4., (PerkinElmer Inc.). Regions of interest were selected using Phenochart, version 1.0.9. (PerkinElmer Inc.) for multispectral imaging at 20 times magnification. InForm software, version 2.2.1. (PerkinElmer Inc.) was used for spectral unmixing of Opal fluorophores, DAPI and autofluorescence and downstream imaging analysis.

For the pSTAT3 analysis, the percentage nuclei containing pSTAT3 was counted separately by two investigators. Divergent results were discussed to reach consensus. For the lymphocyte analysis, a selection of 30-35 representative original multispectral images were used to train the inForm software to distinguish tumor tissue from stromal tissue and background based on DAPI and autofluorescence. Settings for adaptive cell segmentation were based on DAPI, and membrane signals. All the settings applied to the training images were saved in an algorithm to allow batch analysis of multiple original multispectral images of all slides.

Image cytometry

Segmented cell data was converted into Flow Cytometry Standard (FCS) files and analyzed using FlowJo software. Immune cells were phenotyped by gating strategy and divided by the surface area of the tissue region (mm^2).

Statistical analysis

The sample size was based on an anticipated 30% immunological response rate for the arm without cisplatin and 70% in the arm with cisplatin. RFS (stage III patients), PFS (stage IV patients), and OS (all patients) were calculated from the date of apheresis to the date of recurrence, progression and death, using the Kaplan-Meier method. Recurrence and progression were censured in case of non-melanoma related death. Difference between

treatment groups was evaluated using a log-rank test. Follow-up duration was determined from date of apheresis to date of last follow-up and censored for death. Paired t-tests were performed to evaluate KLH responses before and after vaccination and independent-samples t-tests were used to evaluate differences in increase in KLH proliferation between groups. For TM^+ $CD8^+$ T cells and functional T cells, differences between groups were evaluated using a Chi-Square test or 2-sided Fisher's Exact test in case of expected count < 5. *P* values less than 0.05 were considered significant. IBM SPSS Statistics for Windows, version 25.0 (IBM Corp., Armonk, NY) and Graphpad Prism 5.03 (GraphPad Software Inc., San Diego, CA) were used for statistical analysis and data visualization.

Results

Patient and vaccine characteristics

Sixty patients were screened and included in the trial (**Supplementary Figure 2**) of whom 6 were replaced. Two stage IV patients because no acceptable DC product could be produced and in 4 stage IV patients since they were not immunologically evaluable due to progressive disease prior to completion of the first cycle. Therefore, 54 patients were included in the final analysis: 22 stage III and 32 stage IV melanoma patients. They were randomly assigned to receive either DC vaccination alone or DC vaccination combined with cisplatin 50 mg/m^2. In 53 out of the 54 included patients a DC product meeting the predefined phenotypic minimal release criteria of mature DCs used in clinical trials could be produced from the first apheresis (**Supplementary Figure 3A**). In the remaining patient this was achieved after a repeated apheresis. Flow cytometry confirmed intracellular protein expression of both gp100 and tyrosinase in DCs of the DC products (**Supplementary Figure 3B**). In two patients, yield was not sufficient for 3 vaccinations, therefore apheresis was repeated during the first cycle.

Baseline characteristics of all immunologically evaluable patients are summarized in **Table 1**. Overall, in the stage III group, 5 patients (23%) had stage IIIA, 6 (27%) had stage IIIB, and 10 (45%) had stage IIIC disease. Of one patient the substage was unknown (IIIX) because the primary tumor had not been assessed, therefore information on ulceration was lacking. Most (73%) patients with IIIC melanoma were randomized to receive monotherapy. Ten stage III patients (45%) completed all 3 cycles of 3 vaccinations; 6 patients receiving combination therapy and 4 patients with DC monotherapy. Twelve stage III patients discontinued the study prior to the completion of the treatment phase due to recurrent disease.

The stage IV group included 26 patients (81%) with advanced cutaneous melanoma, 2 (6%) with irresectable stage III disease, and 4 patients (13%) with metastatic uveal melanoma. Of the stage IV patients only 5 patients (19%) completed 2 cycles and thereof 2 (9%) completed the total of 3 cycles of vaccinations. One stage IV patient in the combination group with stable disease at the first evaluation scan at 3 months, was referred for palliative surgical resection of a stable ileal metastasis to lower the risk of a gastrointestinal bleeding. In all stage IV patients who did not complete all cycles, vaccinations were stopped due to progressive disease. At clinical data cutoff April 23th 2019, median follow-up time from apheresis was 62.3 months in stage III and 64.9 months in stage IV patients.

Table 1 Baseline characteristics

	Stage III melanoma patients		Stage IV melanoma patients	
	DC vaccination (n = 11)	DC vaccination + cisplatin (n = 11)	DC vaccination (n = 16)	DC vaccination + cisplatin (n = 16)
Sex, n (%)				
Male	9 (82)	9 (82)	8 (50)	10 (63)
Female	2 (18)	2 (18)	8 (50)	6 (38)
Age				
Median (range) (years)	53 (25-69)	48 (25-67)	61 (34-69)	54 (30-69)
HLA-A2.1, n (%)				
Positive	7 (64)	9 (82)	5 (31)	9 (56)
Negative	4 (36)	2 (18)	11 (69)	7 (44)
Site of primary melanoma, n (%)				
Skin	10 (91)	10 (91)	12 (75)	12 (75)
Eye	0 (0)	0 (0)	3 (19)	1 (6)
Unknown primary	1 (9)	0 (0)	1 (6)	3 (19)
Primary not assessed	0 (0)	1 (9)	0 (0)	0 (0)
AJCC stage (7th edition)[a], n (%)				
IIIA	2 (18)	3 (27)	n.a.	n.a.
IIIB	1 (9)	4 (36)		
IIIC	8 (73)	3 (27)		
IIIX	0 (0)	1 (9)		
Adjuvant radiotherapy, n (%)				
No	7 (64)	8 (73)	n.a.	n.a.
Yes	4 (36)	3 (27)		
M stage at inclusion, n (%)				
M0	n.a.	n.a.	1 (6)	1 (6)
M1a			3 (19)	4 (25)
M1b			5 (31)	4 (25)
M1c			7 (44)	7 (44)
Prior treatment for stage IV disease, n (%)				
No	n.a.	n.a.	7 (44)	12 (75)
Surgery			8 (50)	3 (19)
Radiotherapy			1 (6)	0 (0)
Targeted therapy			1 (6)	0 (0)
Chemotherapy			1 (6)	0 (0)
Regional perfusion			0 (0)	2 (13)

[a] The appropriate American Joint Committee on Cancer (AJCC) TNM system was used for both cutaneous (7th edition [21]) and uveal (7th edition [22]) melanomas.

Adverse events

The safety analysis included all patients who completed at least one cycle of vaccinations (n = 54) (**Table 2**). The 4 patients who did receive at least 1 DC vaccine, but did not complete at least one cycle, showed no striking features in their toxicity profile. In the combination group, frequent adverse events included nausea and fatigue. One treatment-related grade 3 adverse event (4%) occurred, consisting of decompensated heart failure due to fluid overload. Cisplatin was stopped because of adverse events in 4 patients (15%) based on decompensated heart failure after vaccination 1, a deep venous thrombosis after vaccination 2, persistent grade 2 tinnitus after vaccination 5, and grade 2 nausea and fatigue after vaccination 7. DC vaccination was continued as monotherapy in all 4 patients. The dose of cisplatin was reduced in 1 patient after vaccination 5 because of grade 2 nausea. Treatment-related adverse events leading to dose interruptions occurred in 2 patients treated with cisplatin; in one patient due to grade 2 tinnitus and in the other patient due to grade 2 thrombocytopenia. In the group treated with DC monotherapy, the most frequent adverse events were flu-like symptoms and injection site reactions. Flu-like symptoms lasted usually less than 48 hours. No grade 3-4 events were seen in patients treated with DC monotherapy.

Table 2 Treatment-related adverse events

	DC vaccination (n = 27)		DC vaccination + cisplatin (n = 27)			*p*-value
	Grade 1	Grade 2	Grade 1	Grade 2	Grade 3	
	Number of events (%)					
Any event	22 (81)	2 (7)	18 (67)	7 (26)	1 (4)	0.159
Injection site reaction	20 (74)	1 (4)	10 (37)	0	0	0.008
Flu-like symptoms	19 (70)	3 (11)	17 (63)	0	0	0.092
Nausea	4 (15)	0	15 (56)	3 (11)	0	<0.001
Vomiting	4 (15)	0	1 (4)	3 (11)	0	0.091
Creatinine increased	2 (7)	0	3 (11)	0	0	0.639
Constipation	0	0	7 (26)	0	0	0.005
Fatigue	0	0	4 (15)	3 (11)	0	0.018
Tinnitus	0	0	3 (11)	2 (7)	0	0.064

Adverse events that occurred in at least 10% of patients and were classified as possibly, probably or definitely related to the treatment by the investigator are depicted.

Induction of de novo immune responses

DCs were loaded with the control antigen KLH, to test the capability to induce de novo immune responses upon DC vaccination. PBMCs, before start of therapy and after consecutive vaccinations of the first cycle, were available for 53 patients (98%) and analyzed for the occurrence of KLH-specific T cell responses. An increase in KLH-specific

T cell proliferation was found in all patients and no significant difference in mean increase was found between both treatment groups (p = 0.453; **Figure 1A**). In addition, no significant difference in mean increase was seen between stage III and IV melanoma patients (data not shown).

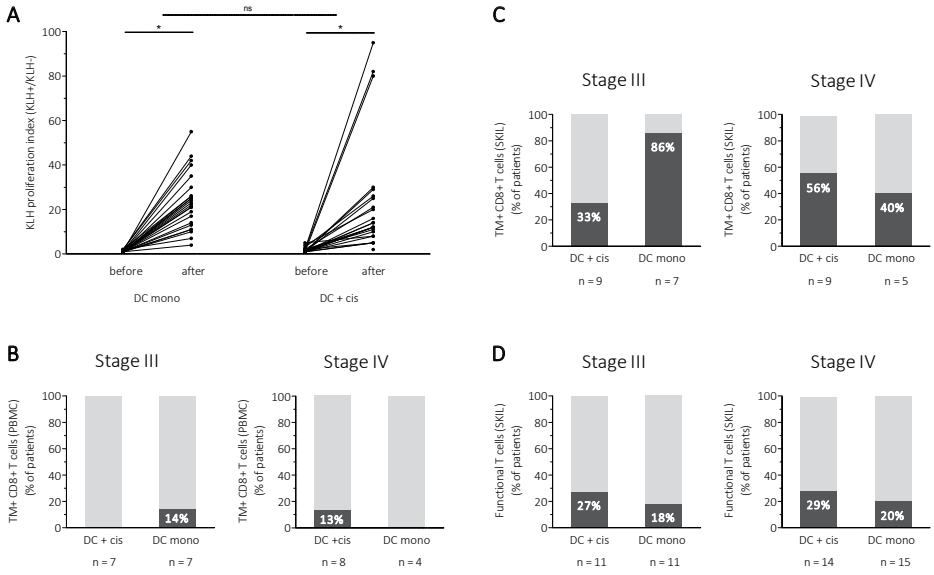

Figure 1 Immunological responses

(**A**) KLH-specific T cell proliferation was measured before the start of therapy and after each vaccination of the first cycle in PBMCs of melanoma patients. The proliferative response to KLH is depicted as a proliferation index (proliferation with KLH/proliferation without KLH). (**B**) PBMCs were tested for TM^+ $CD8^+$ T cells recognizing gp100 or tyrosinase in HLA-A2.1 positive patients. (**C**) SKILs were tested for TM^+ $CD8^+$ T cells recognizing gp100 or tyrosinase in HLA-A2.1 positive patients and (**D**) a functional T cell response in all patients. * $p < 0.001$, *DC* dendritic cell, *KLH* Keyhole Limpet Hemocyanin, *ns* not significant, *PBMC* peripheral blood mononuclear cell, *SKIL* skin-test infiltrating lymphocytes, *TM* tetramer

Induction of tumor antigen-specific T cells

The presence of gp100- and tyrosinase-specific $CD8^+$ T cells recognizing at least one of the two antigens was tested by HLA-A2.1 tetrameric MHC-peptide complexes in both PBMC and T cells cultured from biopsies of DTH injection sites (SKILs). This test was performed in the 30 HLA-A2.1 positive patients, of which 18 received combination therapy and 12 DC monotherapy. After the first cycle, TM^+ $CD8^+$ T cells were found in PBMCs of 2 patients, 1 in each treatment group (**Figure 1B**).

TM$^+$ CD8$^+$ T cells were found more frequently in SKILs, in 16 of 30 HLA-A2.1 positive patients (53%) (**Figure 1C**). No difference was seen between treatment groups: 44% in the combination and 67% in the monotherapy group. In HLA-A2.1 positive stage III patients, 33% treated with combination therapy compared to 86% treated with DC monotherapy showed TM$^+$ CD8$^+$ T cells in their SKILs. In HLA-A2.1 positive stage IV patients slightly more patients in the combination group (56%) compared to the monotherapy group (40%) showed TM$^+$ CD8$^+$ T cells in their SKILs . No difference was seen, regardless of the treatment arm, between all HLA-A2.1 positive stage III (56%) and stage IV patients (50%).

SKILs were analyzed for the occurrence of a functional T cell response, by measuring production of IFNγ in response to relevant target cells loaded with gp100 or tyrosinase in HLA-A2.1 positive patients, and by using antigen-loaded autologous EBV-transformed B cells in HLA-A2.1 negative patients. No difference in the presence of functional T cells in patients treated with combination therapy (28%) and patients treated with DC monotherapy (19%) was found (**Figure 1D**).

STAT expression and T cell infiltrate in tumor tissue

Of patients with available tumor tissue, tumor samples prior to and after DC vaccination were compared. Samples were not derived from the same tumor site, although we took into account that tissue of the same organ of origin was used, to obtain the best possible comparability. Nuclear pSTAT3 expression was estimated as the percentage of nuclei with pSTAT3 expression from the total number of nuclei. There were no clear differences in pSTAT3 changes prior and after DC vaccination between patients treated with monotherapy compared to patients receiving combination therapy (**Supplementary Figure 4A-B**). Therefore, we investigated more possible effects of cisplatin, such as decreasing numbers of Tregs in the TME.[13, 17] With multiplex immunofluorescence we investigated the presence of CD8$^+$ T cells, CD4$^+$ T cells and FoxP3$^+$ cells. In these samples, no differences were observed between patients treated with or without cisplatin (**Supplementary Figure 4C-E**).

Due to the retrospective nature of this analysis, only paired tissue samples were available of patients with recurrent or progressive disease. To circumvent this drawback, we also analyzed PBMCs for the presence of M-MDSCs and Tregs prior to the start and after 3 cycles of vaccinations, as this was also available for patients without recurrent disease. We found no difference in the presence of M-MDSCs or Tregs between stage III melanoma patients treated with or without concomitant cisplatin (**Supplementary Figure 5**).

From one patient with a partial response to combined treatment of DC vaccination with cisplatin, sequential tumor biopsies from clinically responding cutaneous metastases were taken (**Figure 2**). This patient was HLA-A2.1 negative and did not show a functional T cell response in the DTH biopsies, but showed clear development of tumor necrosis and expanding T cell infiltration in tumor biopsies taken during treatment.

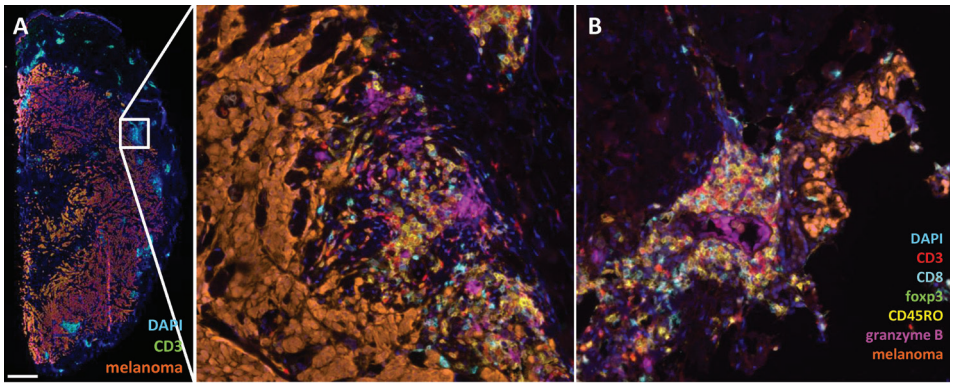

Figure 2 T cell infiltration in melanoma metastases
Multispectral images of cutaneous metastases were taken using the Vectra slide scanner at the start and during treatment in a patient with a partial response to dendritic cell vaccination in combination with cisplatin. Panel **A** includes a biopsy at the start of treatment. The overview image shows extensive melanoma cells with groups of $CD3^+$ cells. When zoomed in (middle image), it reveals that $CD8^+$ T cells and granzyme B were present at the start of treatment. Panel **B** shows an image of a clinically responding cutaneous metastasis after the second cycle of vaccinations combined with cisplatin; only few melanoma cells were detected while an extensive T cell infiltrate was found, including $CD8^+$ T cells and $CD45RO^+$ cells.

Clinical response

Median RFS of stage III patients in the combination treatment group was 45.9 months versus 9.6 months in the monotherapy group (p = 0.245; **Figure 3A**). The 2-year RFS rates in the combination group and monotherapy group were 64% and 27% at 2 years. The median OS of stage III patients treated with cisplatin was not reached, as compared with 32.0 months in stage III patients without cisplatin (p = 0.012; **Figure 3B**). The 2-year OS rates were 82% and 64% in the combination and monotherapy group, respectively. One patient in the monotherapy group died because of a non-melanoma related cause without evidence of recurrent disease.

Median PFS of stage IV melanoma patients in the combination group was 4.7 months, as compared to 3.0 months in the monotherapy group (p = 0.101; **Figure 3C**). When excluding metastatic uveal melanoma patients (1 patient in the cisplatin group and 3 in the monotherapy group), median PFS was 5.4 months in the combination group versus 3.5 months in the monotherapy group (p = 0.121). Stage IV melanoma patients treated with monotherapy had a trend towards a longer OS with a median OS of 19.0 months versus 12.2 months in patients in the combination group (p = 0.063; **Figure 3D**). However, when excluding patients with uveal melanoma this trend diminishes, as shown by a median OS of 19.0 versus 12.9 months (p = 0.108), respectively. Subsequent treatment may have caused differences in OS; compared to the combination group, more patients with progressive disease in the monotherapy group received treatment with ipilimumab (69% versus 31%) or with anti-PD1 antibodies (38% versus 0%).

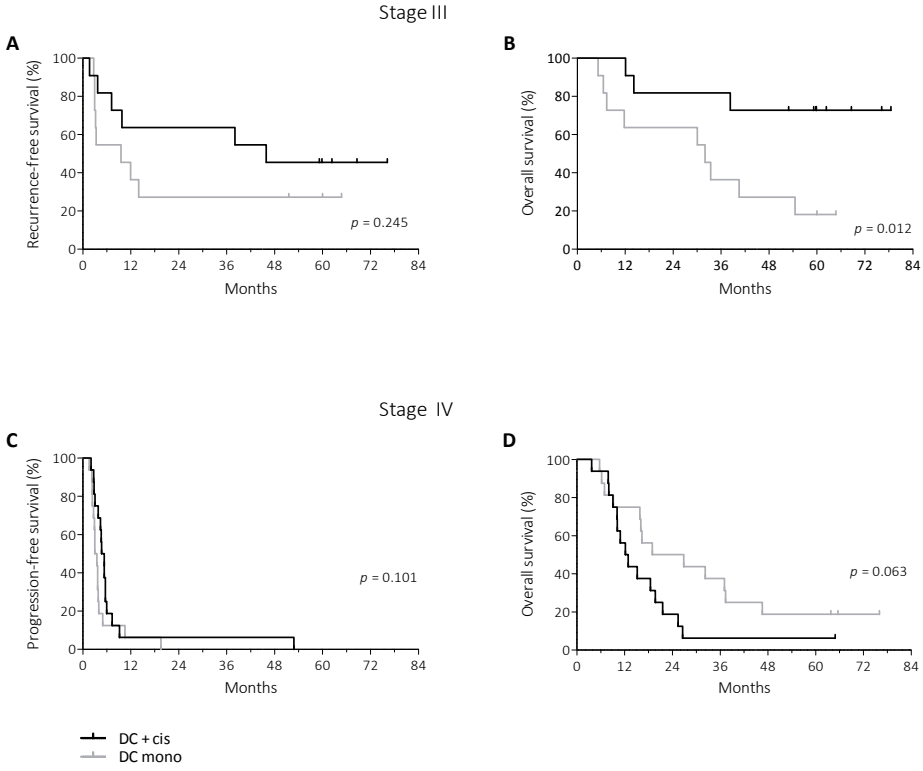

Figure 3 Survival curves in stage III and IV melanoma patients

Kaplan-Meier curves for (**A**) recurrence-free survival and (**B**) overall survival in stage III melanoma patients. For stage IV melanoma patients, Kaplan-Meier curves for (**C**) progression-free survival and (**D**) overall survival are shown

Discussion

In this randomized phase 2 trial, we showed combination of autologous DC vaccination with cisplatin is feasible and safe. Viable DCs, which met the previously determined minimal release criteria[26], could be produced in 95% of patients. The toxicity profile of the combination treatment showed no unexpected safety concerns. Adverse events leading to discontinuation, treatment interruption or dose reduction of cisplatin took place in a minority of patients in the combination group. As expected, grade 1-2 local injection site reactions and flu-like symptoms were common adverse events in both treatment groups, but occurred less in the combination group. These side effects might have been suppressed by dexamethasone, which was given as an antiemetic drug in patients receiving cisplatin.

We could not confirm our hypothesis that the immunomodulatory effect of cisplatin improves the immunological response rate of DC vaccination when combined. In both treatment groups, DC vaccines were able to induce de novo immune responses, as patients in both groups showed a cellular response against the control antigen KLH. TM^+ $CD8^+$ SKILs were induced in about half of the patients without difference between the treatment groups, but in stage III melanoma patients, combination therapy might have induced less TM^+ $CD8^+$ T cells than monotherapy.

The lack of improved immunological response to the combination of DC vaccination and cisplatin might be explained by several reasons. It is possible that the immunomodulatory effects of cisplatin are not strong enough to induce a significant difference in the number of T cell responses *in vivo*. Although, we saw a clear increase in T cell infiltration in the metastasis of one responding patient to the combined treatment, we found little other evidence on the *in vivo* immunostimulatory effect of cisplatin. We did not find a downregulation in the expression of nuclear pSTAT3. The timing of tumor sampling relative to the dosing of cisplatin might be suboptimal, as the interval between the last dose of cisplatin and retrieval of tumor tissue was at least a few weeks. And in earlier reports the *in vitro* effects on STAT expression were seen immediately after exposure to platinum drugs.[14, 16] In addition, we investigated more possible effects of cisplatin on the composition of the immune infiltrate, which likewise showed no clear difference between treatment groups. A possible reason for not finding any difference could lie in the retrospective nature of the collection of tumor material, consequently we solely analyzed patients with progressive or recurrent disease. As pSTAT3 upregulates with tumor proliferation, this is a likely explanation of the increased pSTAT3 expression after vaccination in all but two patients (one in each group). It would have been interesting to prospectively collect tumor biopsies prior to and during experimental treatment for better comparability of both responders and non-responders.

Furthermore, dexamethasone could have had a negative effect on DC vaccination in the combination group. Glucocorticosteroids might decrease the number of circulating $CD8^+$ and $CD4^+$ T cells and increase the proliferation of Tregs.[30, 31] Therefore, dexamethasone might decrease both the sensitivity of our immunomonitoring tests and the positive additive effects of cisplatin on the anti-tumor immune response. However, the effect of dexamethasone on the outcome of treatment with immunotherapy has not been clearly established yet. Also, the timing of the DC vaccination in relation to the cisplatin might have caused the lack of synergy. In contrast to our study, Welters and colleagues found that chemotherapy (6 cycles carboplatin-paclitaxel every 3 weeks) resulted in vigorous vaccine-induced T cell responses in advanced cervical cancer patients when a single dose of HPV16-synthetic long peptide vaccine was given 2 weeks after the second cycle of chemotherapy.[32] Their study showed that the effect of chemotherapy on decreasing circulating myeloid cells was most pronounced starting 2 weeks after the second cycle of chemotherapy, resulting in an optimal immunological window for vaccination. Of course, there are differences in tumor type, chemotherapy, and type of vaccines, but the most important difference might be the time interval between chemotherapy and vaccinations. In our study, vaccinations and chemotherapy were both initiated in the first cycle and administered subsequently on the same day, where later start of vaccinations might have been better.

As for the absence of an improved immunological response rate, we also could not find a clear positive effect of the combination treatment on survival. A significant better OS is observed in stage III patients treated with DC vaccination and cisplatin compared to patients treated with DC vaccination monotherapy. However, groups are too small and heterogenous to draw firm conclusions, and the data is not supported by a significant improvement in RFS. In addition, the difference in OS might be caused by ongoing responses to ICI in 2 patients in the combination group. In contrast, in the monotherapy group none of the relapsed patients responded to ICI. Finally, in the stage IV patients, no significant difference in PFS and OS was found. The stage IV patients even showed a trend towards a better OS in the DC monotherapy arm. Again, this is most likely explained by subsequent treatment and not by the study treatment. Taken together, we cannot conclude that the addition of cisplatin to DC vaccination is of benefit to stage III or IV melanoma patients.

This trial was initiated before ICI were available, but today a study combining DC vaccination with ICI would be preferred over investigating the combination with chemotherapy.[33] This combination might intensify proliferation and effector functions of tumor-specific T cells induced by DC vaccination by blocking immune checkpoints with anti-CTLA-4 or anti-PD-1 antibodies.[34, 35] A recent phase 2 trial with autologous DC combined with ipilimumab showed tolerability and an encouraging objective response rate (38%) in pre-treated advanced melanoma patients.[36] Further clinical trials, mainly with the less toxic and more effective anti-PD-1 antibodies, are under investigation in multiple tumor types and results are awaited.[37]

In subsequent trials with stage III melanoma patients, it is also important to restage patients before trial participation to exclude distant metastases. We recently showed that 18% of stage IIIB/C patients have a relapse as early as prior to the start of adjuvant therapy, despite preoperative exclusion of distant metastases.[38] In this trial 91% of stage III patients were not restaged after prior negative screening for distant metastases and thus this likely increased heterogeneity in our stage III patients as undiscovered relapses influences RFS rates.

In conclusion, the combination of autologous monocyte-derived DC vaccination and cisplatin in stage III and IV melanoma patients is feasible and safe, but does not seem to result in more tumor-specific T cell responses or clinical benefit when compared to DC monotherapy. Together with the currently available ICI for the treatment of stage III and IV melanoma patients, these results do not appear to justify the use of cisplatin in combination with DC vaccination in melanoma patients. Future research should focus on the more promising combination of DC vaccination with ICI.

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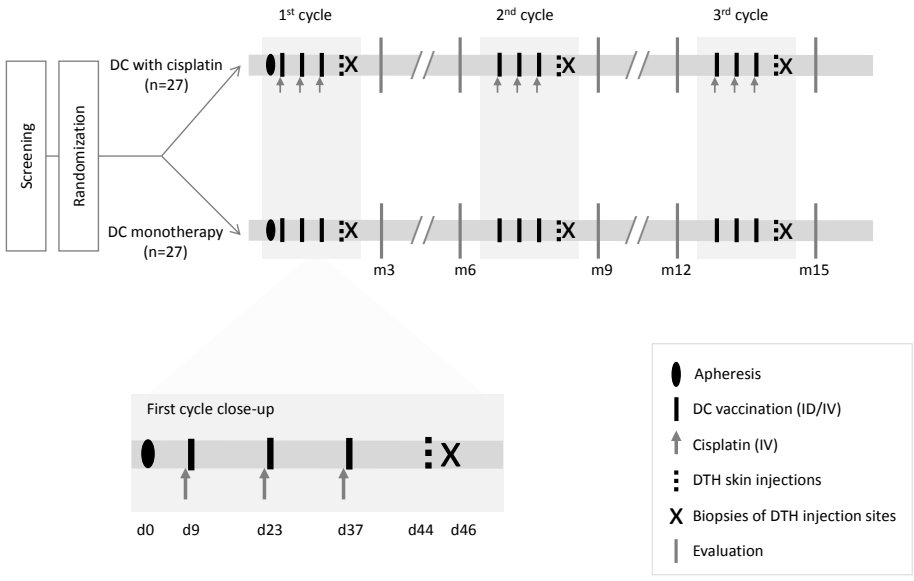
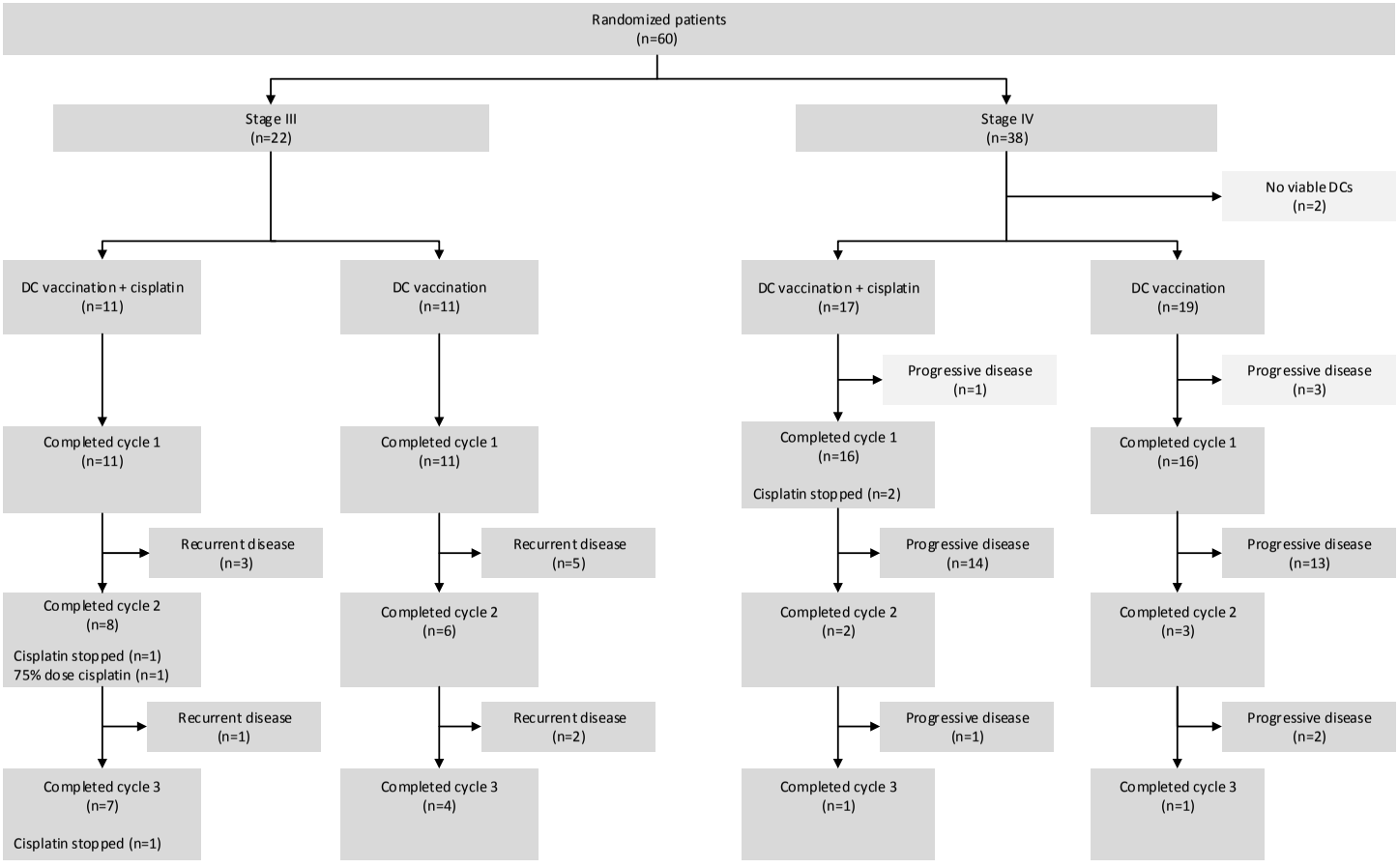
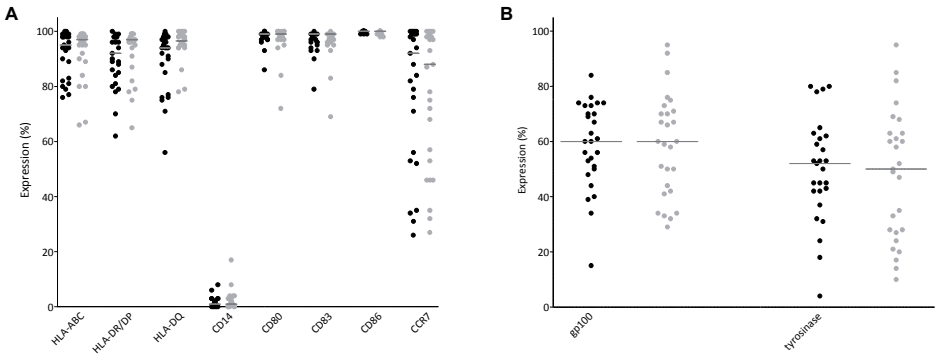
Supplementary Material



Supplementary Figure 1 Study schedule during the treatment phase
Patients were randomized between DC vaccination with cisplatin and DC vaccination monotherapy. Standard evaluation in stage IV patients consisted of CT of chest and abdomen and in stage III patients of medical history and physical examination. *D* day, *DC* dendritic cell, *DTH* delayed type hypersensitivity, *ID* intradermally, *IV* intravenously, *m* month

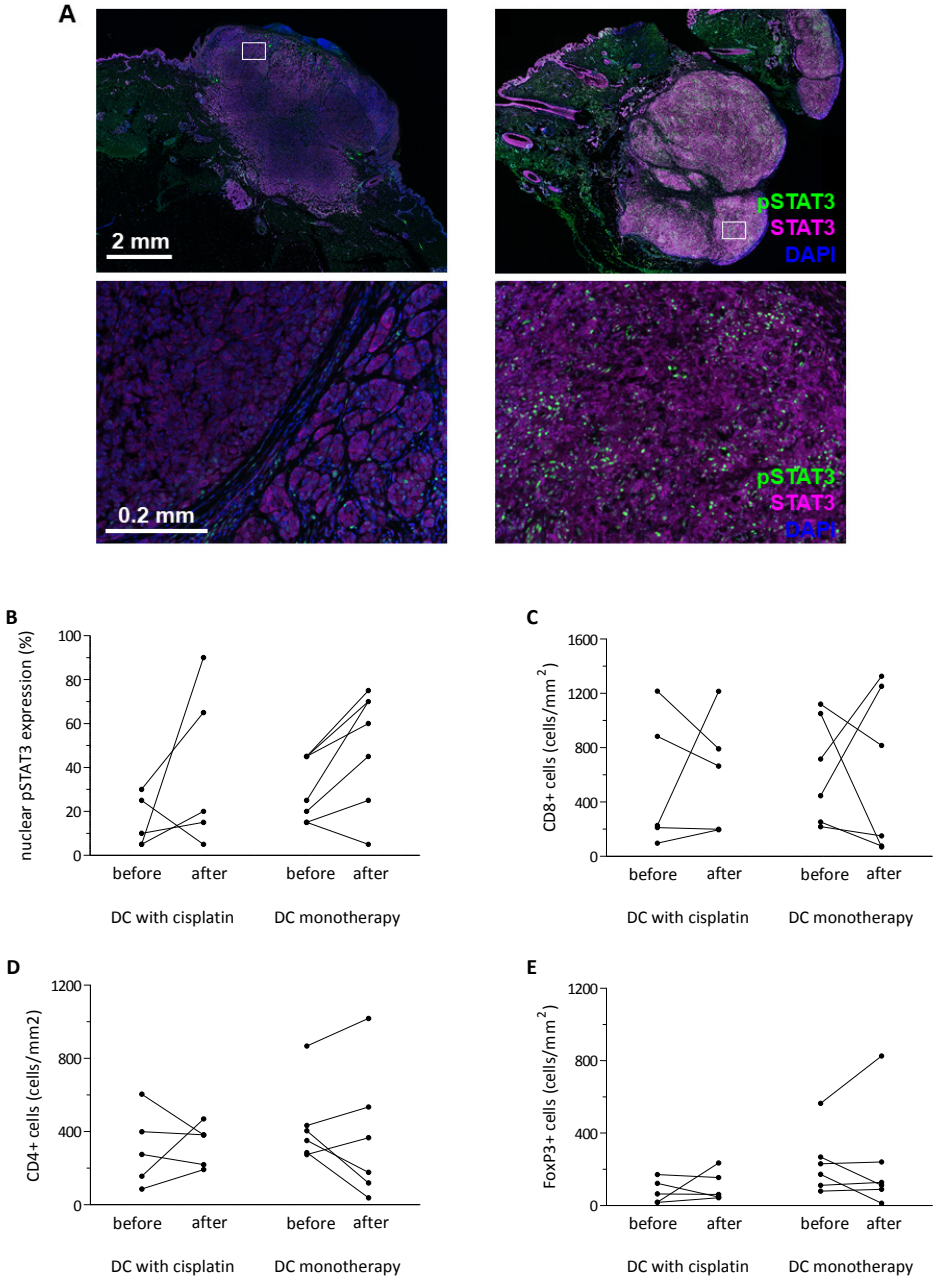


Supplementary Figure 2 Flow chart of all randomized stage III and IV melanoma patients



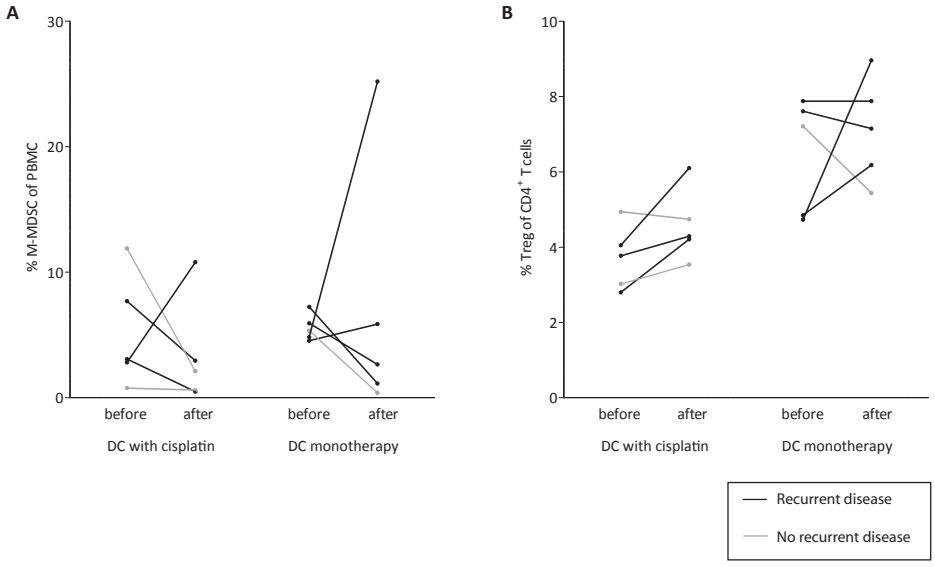
Supplementary Figure 3 Vaccine characteristics

Expression patterns of the *ex vivo* generated dendritic cells used in the first cycle. Shown are the percentages of dendritic cells expressing the (**A**) different phenotype markers and (**B**) tumor-associated antigens. The lines represent the median.



Supplementary Figure 4 pSTAT3 expression and infiltrate in melanoma tissue

Analysis of melanoma tissue prior to and after experimental therapy. (**A**) Representative image of a pSTAT3 staining by immune histochemistry, shown are images of (**left**) low and (**right**) high expression. (**B**) Expression of nuclear pSTAT3. Analysis of lymphocyte infiltrate with absolute numbers of (**C**) $CD8^+$, (**D**) $CD4^+$ cells and (**E**) $FoxP3^+$ cells per mm^2.



Supplementary Figure 5 Monocytic myeloid-derived suppressor cells and regulatory T cells in peripheral blood.

Peripheral blood mononuclear cells (PBMCs) were collected prior to the apheresis (start of study) and one week after the third vaccination. (**A**) percentage of monocytic myeloid-derived suppressor cells (HLA-DR$^-$CD14$^+$CD11b$^+$CD33$^+$) of live PBMCs and (**B**) the percentage regulatory T cells (Tregs) of CD4$^+$ T cells was determined for stage III patients treated with dendritic cell vaccination with or without concomitant cisplatin.

3

Stage III melanoma patients respond to adjuvant vaccination with combined CD1c$^+$ myeloid and plasmacytoid dendritic cells

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Abstract

Purpose. To evaluate the immunological responses of lymph-node involved (stage III) melanoma patients to adjuvant dendritic cell vaccination with subsets of naturally occurring dendritic cells (nDCs).

Patients and methods. Fifteen patients with completely resected stage III melanoma were randomized to receive adjuvant dendritic cell vaccination with $CD1c^+$ myeloid dendritic cells (mDCs), plasmacytoid dendritic cells (pDCs) or the combination. Immunological response was the primary endpoint and secondary endpoints included safety and survival.

Results. In 80% of patients antigen-specific $CD8^+$ T cells were detected in skin test-derived T cells and in 55% of patients, antigen-specific $CD8^+$ T cells were detectable in peripheral blood. Functional interferon-γ-producing T cells were found in the skin test of 64% of patients. Production of nDC vaccines meeting release criteria was feasible for all patients. Vaccination only induced grade 1-2 adverse events, mainly consisting of fatigue.

Conclusion. Adjuvant dendritic cell vaccination with mDCs and/or pDCs is feasible, safe and induced immunological responses in the majority of stage III melanoma patients.

Introduction

As dendritic cells (DCs) are the most potent antigen-presenting cells, presenting antigens to naive T cells, they play a pivotal role in the induction of adaptive immune responses against tumors.[1] For DC vaccination of cancer patients, autologous DCs are matured and loaded with the relevant tumor antigens *ex vivo* and are subsequently administered to the patient to induce a tumor-specific T-cell responses *in vivo*.[2] Because the T cell activation is highly antigen-specific, the toxicity profile of DC vaccination is mild.[3]

Recent major breakthroughs in immunotherapy in cancer patients mainly consist of clinical benefit from immune checkpoint inhibitors (ICI). Unfortunately, and in contrast to DC vaccination, these drugs can give rise to serious immune-related toxicity due to the enhancement of non tumor-specific immune responses against healthy cells.[4] To date, survival benefit with DC vaccination has not been established. However, in proof of principle trials DC vaccination induced functional tumor-specific T-cell responses and long-lasting clinical responses.[3] Together with the current knowledge that immunotherapy is able to induce long-term survival benefit and the favorable toxicity profile of DC vaccination, optimization of DC vaccination is an important focus of cancer research.

Until recently, most studies with DC-based immunotherapy were performed with autologous DCs *ex vivo* differentiated from monocytes or CD34+ progenitors. However, the potency of these so-called monocytes-derived DCs (moDCs) may be hampered by their extensive culture period of 5-9 days with cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4 that is needed to differentiate the cells into DCs. Especially, IL-4 potentially reduces the migration capacity of DCs.[5-7] A few years ago, direct isolation of the scarce naturally circulating DCs (nDCs) from blood became possible, thereby omitting this intensive culture period used for the production of moDCs.[8] After direct isolation, nDCs are prepared for vaccination by maturation and antigen loading within two to three days. The two major subsets of nDCs are myeloid DCs (mDCs, also called 'conventional' or 'classical' DCs) and plasmacytoid DCs (pDCs). The major subset of mDCs, cDC2, is characterized by CD11c and CD1c (BDCA-1). A minor population, cDC1, is CD11c and CD141 (BDCA-3) positive. PDCs express both CD303 (BDCA-2) and CD304 (BDCA-4).[9] Vaccination with CD1c+ mDCs or pDCs has been tested in clinical trials in metastastic (stage IV) melanoma and metastastic prostate cancer patients. At time of writing, results of two trials were published and showed that in stage IV melanoma patients, vaccination with pDCs or CD1c+ mDCs led to immunological responses and, in some patients, to long-term survival.[10, 11]

Since the conduction of these trials with nDCs in stage IV melanoma, we made multiple potential advances in the production of the nDC vaccines which are incorporated in our trial. First, the mode of maturation for both DC subsets is optimized. DC maturation is crucial for proper T cell activation.[12-14] Especially, exposure of DCs to pathogen-associated molecular patterns, such as Toll-like receptor ligands, yields DCs that induce potent T helper 1 and

cytotoxic T cell responses.[15-18] MDCs and pDCs express Toll-like receptors 7 and 8, respectively, which can be triggered by single-stranded RNA (ssRNA). We have shown that complexes of ssRNA stabilized with protamine (pR) can activate both CD1c$^+$ mDCs and pDCs into functional mature DCs, secreting IL-12 and interferon (IFN)α, respectively.[19] Second, we showed that a CD1c$^+$ mDC subpopulation expressing both CD1c and the monocytic marker CD14, attenuates the induction of T cell responses.[20] Therefore, in our trial, we depleted CD14$^+$ cells prior to the positive selection of CD1c$^+$ mDCs. Third, we expanded the pool of antigens used to load the DCs. We added the cancer-testis antigens (CTA) MAGE-C2, MAGE-A3 and NY-ESO-1 to the previously used tumor-associated antigens (TAA) gp100 and tyrosinase. Both these CTA and TAA are known to be frequently expressed in melanoma tissue.[21, 22] Besides, antigens binding HLA types other than HLA-A2.1 were added, to enable induction of immunological responses in HLA-A2.1 negative patients. In addition to a broader panel of HLA-restricted peptides, we added peptide pools with overlapping peptides that cover the complete antigen sequence and bind multiple HLA types, both major histocompatibility complex (MHC) class I and II.

Besides the aforementioned changes that potentially improved mDC and pDC vaccinations compared to previous trials, we assessed the administration of CD1c$^+$ mDCs and pDCs simultaneously. When combined, CD1c$^+$ mDCs and pDCs might have a synergistic effect, as they possess a distinct phenotype, capacity in pathogen detection and produce different cytokines.[23, 24] In a murine model, vaccination with combined mDCs and pDCs was superior in reducing tumor size and increased survival compared to either one of the subsets alone.[25] Therefore, combining both subsets might further enhance the induction of an immunological and clinical response.

We investigated vaccination with these improved and combined CD1c$^+$ mDCs and pDCs in lymph-node involved (stage III) melanoma patients. Cutaneous melanoma is an aggressive form of skin cancer due to its metastatic potential. Compared to patients with stage IV melanoma, patients with completely resected stage III melanoma harbor less tumor burden, hence less tumor-induced immune suppression[26] which might hamper response to DC vaccination. As such, DC vaccination might be more successful in stage III melanoma patients. This is endorsed by superior induction of antigen-specific T cells by DC vaccination in stage III compared to stage IV melanoma patients.[27, 28] In addition, a retrospective analysis of stage III melanoma patients receiving adjuvant moDC vaccination showed improved overall survival (OS) compared to their matched controls.[29] Stage III melanoma is treated with surgical resection with curative intent. Unfortunately, despite complete surgical resection, patients have a high risk of recurrence resulting in 5-year OS rates between 40 and 78%.[30] Therefore, effective adjuvant therapy for this group of patients is warranted. At time of trial enrollment, no adjuvant therapy significantly impacting survival was registered and for this reason we included patients with completely resected stage III melanoma to receive adjuvant nDC therapy. In the meantime, after completion of the enrollment phase, several drugs were approved for use as an adjuvant therapy.[31-34]

Here we present the results on immunological response, feasibility and safety in our randomized phase II trial including stage III melanoma patients receiving adjuvant DC vaccination with CD1c+ mDCs, pDCs or the combination of both (combiDCs). This is the first clinical trial with combiDCs in melanoma patients.

Patients and methods

Clinical protocol

Fifteen patients with stage III cutaneous melanoma, according to the 7th edition of the American Joint Committee on Cancer (AJCC) staging system[30], were included. Patients were randomly assigned on a 1:1:1 basis to receive mDCs, pDCs or combiDCs. The primary endpoint was immunological response to single and combined mDC and pDC vaccination. Safety, recurrence-free survival (RFS), OS and health-related quality of life assessment (HRQoL) were secondary objectives. This trial (NCT02574377) has been approved by the Central Committee on Research Involving Human Subjects and is in concordance with the Declaration of Helsinki as defined by the International Conference on Harmonization.

Eligible patients were 18-75 years of age with histologically documented stage III melanoma, completely removed including a radical lymph node dissection (RLND) within 12 weeks prior to the start of study (apheresis). Other key inclusion criteria were WHO performance score 0 or 1 and a normal serum lactate dehydrogenase. Exclusion criteria included autoimmune diseases, immunosuppressive conditions and any concurrent adjuvant therapy including radiotherapy. Due to the use of keyhole limpet hemocyanin (KLH), patients with a known allergy to shell fish were excluded. Informed consent was obtained from all participants.

Four weeks after an apheresis, the first vaccination of a cycle consisting of three biweekly vaccinations was administered (**Supplementary Figure 1**). This cycle of three vaccinations was repeated twice, with an interval of six months between cycles. Between 1-2 weeks after the third vaccination of each cycle, patients were assessed for their immunological response by skin tests. If patients did not reach the first immunological assessment they were replaced, since this was the primary endpoint of our trial. Patients were followed for up to 5 years with follow-up visits every 3 months during the first 2 years and every 6 months for the last 3 years. Assessment for recurrent disease was performed by medical history, physical examination and, when indicated, by imaging.

Adverse events were scored according to the Common Terminology Criteria for Adverse Events (CTCAE) version 4.0. Adverse events considered as possibly, probably or definitely related to the study drug according to the investigator were considered treatment-related.

Dendritic cell isolation and vaccine preparation

Patients were vaccinated with autologous mDCs and/or pDCs loaded with tumor peptides and overlapping peptide pools (**Figure 1**). Autologous mononuclear cells were harvested by apheresis. PDCs and mDCs were selected with the fully automated and enclosed immunomagnetic CliniMACS Prodigy® isolation system (Miltenyi Biotec, Bergisch Gladbach, Germany) with GMP-grade magnetic bead-coupled antibodies (Miltenyi Biotec) following the manufacturer's guidelines. When patients were randomized for vaccination with both pDCs and mDCs, pDCs were selected with anti-CD304 (BDCA-4) coupled beads first, followed by depletion of $CD19^+$ and $CD14^+$ cells and positive selection of $CD1c^+$ cells. For the single mDC group, $CD1c^+$ ($BDCA\text{-}1^+$) cells were positively selected after depletion of $CD19^+$ and $CD14^+$ cells, and for the single pDC group only the pDC selection step was completed. MDCs were cultured overnight at a concentration of 1.5×10^6 cells/ml with 800IU/ml recombinant human GM-CSF in TexMACS GMP medium (both Miltenyi Biotec) supplemented with 2% human serum (HS) (Sanquin, Amsterdam, the Netherlands) and KLH (Immucothel, Biosyn Arzneimittel GmbH) for immunomonitoring. PDCs were cultured overnight at a concentration of 1.5×10^6 cells/ml with 10ng/ml recombinant human IL-3 in TexMACS GMP medium (both Miltenyi Biotec) supplemented with 2% pooled HS (Sanquin). Cells used for the delayed-type hypersensitivity (DTH) skin test were cultured without KLH. During overnight culturing MACS® GMP-grade PepTivators®, overlapping peptide pools of the CTA MAGE-A3 and NY-ESO-1 (Miltenyi Biotec) covering the sequence of the entire antigen, were added. After overnight culture, the DCs were matured with 10µl/ml premixed protamine HCL (Meda Pharma, Amstelveen, the Netherlands; 10µg)/mRNA (Universitätsklinikum Erlangen, Erlangen, Germany; 5µg) (protamine/mRNA (pR)) for 6 hours. After 3 hours of maturation, viability and phenotyping was assessed and a mix of fourteen peptides of TAA gp100 and tyrosinase and CTA MAGE-C2, MAGE-A3 and NY-ESO-1 (all Leiden University Medical Center, Leiden, the Netherlands) (**Supplementary Table 1**) was added for the last 3 hours of maturation. DCs used for DTH skin injections were either loaded with peptides or overlapping peptide pools. The procedure had to give rise to mature mDCs and pDCs meeting the following release criteria after 6 hours of maturation: > 50% viability and > 50% CD83 (mDC) and/or > 50% CD80 (pDC) expression. One vaccine consisted of 2-5 $\times 10^6$ mDCs and/or 1-3 $\times 10^6$ pDCs. Cells were cryopreserved in TexMACS medium containing 10% dimethyl sulfoxide (DMSO; WAK chemie Medical GmbH, Steinbach, Germany) and 40% Albuman (Sanquin) and were thawed on the day of administration. For combined pDC and mDC vaccines, both subsets were pooled in one syringe after thawing. Vaccines were checked for sterility prior to clinical application (Eurofins Bactimm, Nijmegen, the Netherlands). An experienced radiologist administered the vaccines intranodally in a clinically tumor-free lymph node under ultrasound guidance. Apheresis was repeated prior to a subsequent cycle if necessary to produce a sufficient number of vaccines.

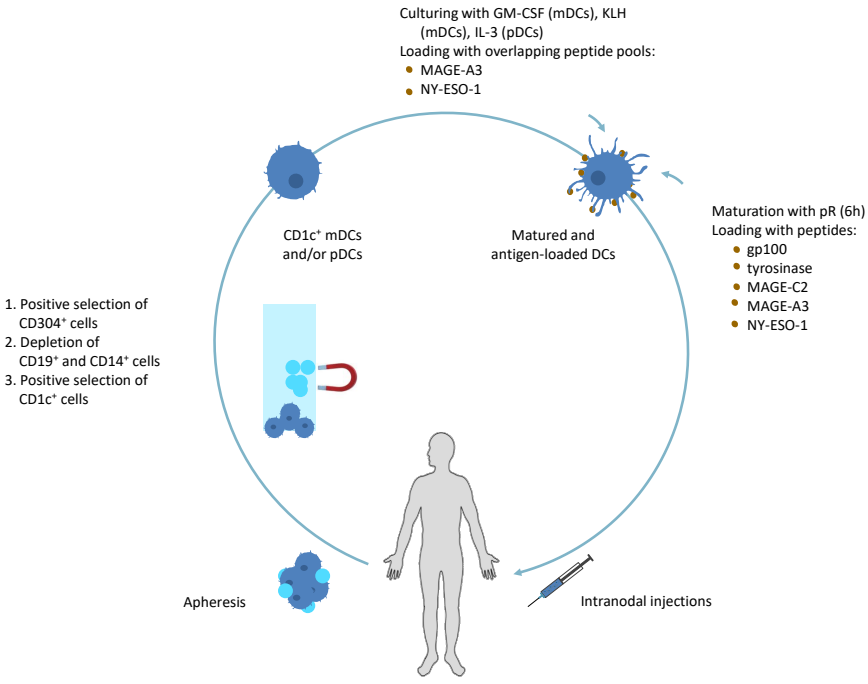


Figure 1 MDC and pDC isolation and vaccine preparation
After mononuclear blood cells apheresis, $CD304^+$ cells (pDCs) were selected. Thereafter, $CD19^+$ and $CD14^+$ cells were depleted followed by $CD1c^+$ cells (mDCs) selection. In the pDC group only pDCs were selected and in the mDC group only the depletion and $CD1c^+$ selection step were performed. After culturing and antigen-loading the DC vaccines were cryopreserved and thawed on the day of intranodal administration. *DCs* dendritic cells, *pR* protamine/mRNA complex, *mDCs* myeloid DCs, *pDCs* plasmacytoid DCs

Tumor antigen-specific and functional T cells

DTH skin tests were performed between 1-2 weeks after the third vaccination of each cycle, as described previously.[35] Depending on randomization, activated mDCs and/or pDCs ($0.1\text{-}0.5 \times 10^6$ cells) were injected intradermally at the back of the patient. Two days after injection, skin punch biopsies (6mm) were obtained from all injection sites to assess the T cell responses. The biopsy specimens were cut in half; one half was cryopreserved and the other half was cut and cultured for 2-5 weeks. After culturing, the skin-test infiltrating lymphocytes (SKILs) were tested for the presence of $CD8^+$ T cells specific for the different tumor antigens used. To analyze occurrence of antigen-specific T cells, SKILs of HLA-A2.1 positive patients were stained with CD8 and tetrameric MHC-peptide complexes (Sanquin) or dextrameric MHC-peptide complexes (Immudex, Copenhagen, Denmark) containing peptides of the relevant as described previously.[35] $CD8^+$ T cells recognizing these MHC-peptide complexes are further referred to as antigen-specific $CD8^+$ T cells. To control for background staining,

samples were stained with tetrameric or dextrameric MHC complexes containing an irrelevant peptide. To test T cell functionality SKILs were cocultured with autologous peripheral blood mononuclear cells (PBMCs) pulsed with the different relevant peptides, overlapping peptide pools, carcinoembryonic antigen (CEA) (negative control) (Leiden University Medical Center,) or no peptide (negative control). Production of IFNγ was measured in the supernatants by cytometric bead array according to the manufacturer's instruction (eBioscience, Vienna, Austria or BD Biosciences, San Jose, CA) after 24 hours of coculture. In addition, PBMCs of HLA-A2.1, HLA-A1 or HLA-B35 positive patients were tested for the presence of antigen-specific $CD8^+$ T cells by staining with tetrameric or dextrameric MHC-peptide complexes as described above.

Flow cytometry

Purity and phenotype of mDCs and pDCs after immunomagnetic isolation were determined by flow cytometry with a FACSVerse® (BD biosciences) or MACS Quant® (Miltenyi Biotec). For this purpose the following primary monoclonal antibodies (mAbs) and the appropriate isotype or fluorescence minus one controls were used: anti-CD1c-Viobright FITC, anti-BDCA2-PE, anti-CD123-APC, anti-CD20-PE-Vio770, anti-CD45-APC-Vio770, anti-CD14-Viogreen, anti-FcεRI-BioBlue, anti-CD14-FITC, anti-CD15-PE, anti-CD56-APC, anti-CD3-BioBlue, anti-HLA-ABC-APC, anti-HLA-DR/DP/DQ-APC, anti-CCR7-APC, anti-CD80-APC, anti-CD83-APC and anti-CD86-APC (all Miltenyi Biotec). After 6 hours of pR stimulation cytokine production of mDCs and pDCs was measured in the supernatant by cytometric bead array according to the manufacturer's instruction (Miltenyi Biotec).

Immunohistochemistry staining of antigen expression

Available tumor tissue of primary melanoma, lymph node metastasis and recurrent disease of the 15 patients was analyzed. To determine antigen expression, we stained for gp100, tyrosinase, NY-ESO-1, MAGE-C2 and MAGE-A. The different MAGE-A antigens are highly homologous[36] leading to cross-reactivity, i.e. T cell receptors recognize multiple members of the MAGE-A antigen superfamily.[37] In addition, the epitopes of the peptides we used to load the DCs are found in different MAGE-A antigens and T cells induced by DC vaccination could therefore potentially also respond to multiple MAGE-A antigens. 4µm slices were cut from formalin fixed paraffin embedded (FFPE) tumor tissue and placed on glass slides. Tissue was deparaffinized in xylene, rehydrated in ethanol and washed in distilled water. Antigen retrieval was performed by boiling in EnVision™ FLEX target retrieval solution (pH 9, K8004, Dako, Santa Clara CA) for 10 minutes for gp100 and tyrosinase samples or in citrate buffer (pH 6, CBB999, ScyTek Laboratories, Logan, UT) for 15 minutes for MAGE-C2, MAGE-A and NY-ESO-1 samples. After cooling down, slides were washed with distilled water and EnVision™ FLEX Wash Buffer (DM831, Dako). Slides were blocked with antibody diluent and afterwards incubated with primary antibodies for the detection of gp100 (M063401, clone HMB45, Dako, dilution: 1/600), tyrosinase (MONX10591, clone T311; Monosan, Uden, the Netherlands; dilution:

1/200), MAGE-A (sc-20034, clone 6C1; Santa Cruz Biotechnology, Dallas, TX; dilution: 1/50), MAGE-C2 (HPA062230, rabbit polyclonal; Merck, Kenilworth, NJ; dilution: 1/200) and NY-ESO-1 (MABC1151, clone D8.38, Merck, dilution: 1/200) for 1 hour. Next, slides were incubated with Opal Polymer HRP (NEL801001KT; PerkinElmer, Waltham, MA) for 30 min. Fluorescent visualization was performed with the Opal 650 (NEL801001KT; PerkinElmer) for 10 minutes. All incubation steps were performed at room temperature. Slides were washed with EnVision™ FLEX Wash Buffer between different incubation steps. Mounting was performed with DAPI Fluoromount-G (0100-20; SouthernBiotech, Birmingham, AL). Slides were prescanned using the PerkinElmer Vectra, version 3.0.4 (PerkinElmer) at 10x magnification. Positivity was scored using whole scanned overviews in Phenochart, version 1.0.9 (PerkinElmer) and 20x regions for representation were selected. For the lymph node tissue the last resected lymph node (harvested during sentinel node biopsy or RLND) containing metastatic disease prior to the start of study was used for staining. Because of the high expression of both gp100 and tyrosinase in melanoma[38], we only assessed the expression of these proteins in patients with recurrent disease. Of these patients, we stained the tissue of the recurrence and the last available material prior to the start of study. Tumor tissue was scored positive for the expression of an antigen when expression was present in at least 1% of tumor cells.

Statistical analysis

The estimates of RFS and OS were calculated using Kaplan-Meier probability. RFS was defined as time from apheresis to first date of recurrence and date of death from any cause was used to calculate OS. When events had not occurred, survival was censored at the date of last follow-up. Follow-up duration was determined from date of apheresis to date of last follow-up and censored for death. To prevent a guarantee-time bias interfering with immunological and clinical responses, we primarily analyzed the data of the immunological response after the first cycle. *P* values of less than 0.05 were considered significant. All statistical analyses were conducted using IBM SPSS Statistics for Windows, version 25.0 (IBM Corp., Armonk, NY). Figures were created with GraphPad Prism, version 5.03 (GraphPad Software, Inc., La Jolla, CA).

Questionnaire

Before and during the study we measured the HRQoL of the participants with the EORTC QLQ-C30 (version 3.0) questionnaire. Results of the HRQoL analysis have been reported separately.[39]

Results

Patients

Between October 2015 and August 2016, 17 patients were included of whom 15 were evaluable. Two patients (pDC-5 and pDC-6) were replaced because of not having reached the first immunological assessment around week 9 due to rapid recurrence. The 15 patients had a median age of 50 (range 19-72) years (for baseline characteristics see **Table 1**). Substages were equally divided over treatment groups, all but 3 patients had stage IIIB or IIIC melanoma. At data cutoff on April 23th 2019, median follow-up was 37.9 months.

Vaccine characteristics

A new apheresis was necessary for 2 patients in the mDC group, due to low purity or viability of the initial mDC product. One patient initially randomized in the combiDC group switched to the mDC group due to low pDC retrieval. Production of DC products meeting release criteria was feasible for all patients (**Supplementary Figure 2A-D**). Median yield, purity and viability were 67 x 10^6, 64% and 87% for mDC products and 48 x 10^6, 62% and 93% for pDC products, respectively. Cytokine production of mDCs and pDCs separately shows that mature mDCs produced IL-12 and mature pDCs produced IFNα, as expected (**Supplementary Figure 2E-F**). All patients received the required 2-5 x 10^6 mDCs and/or 1-3 x 10^6 pDCs in each vaccine. In the thawed vaccines, median viability of DCs was 92% (range 72-99%) for mDCs and 90% (range 59-98%) for pDCs.

Adverse events

All 15 patients experienced at least one grade 1-2 adverse event (**Table 2**), mostly consisting of fatigue. Three patients experienced an injection site reaction, and two patients had flu-like symptoms. During apheresis, 1 grade 3 and 1 grade 4 adverse event were observed, consisting of hypokalemia and hypocalcemia, respectively. These electrolyte disturbances occurred simultaneously in the same patient and are a known possible complication of the apheresis due to interaction with the citrate anticoagulant used during apheresis. Both disturbances were corrected completely with suppletion of electrolytes. The two replaced patients did not experience grade 3 or 4 or any other striking adverse events.

Table 1 Baseline characteristics

Characteristic	Total (n = 15)	MDC group (n = 5)	PDC group (n = 5)	CombiDC group (n = 5)
Age (years)	50	55	48	5
Median (range)	(19-72)	(37-72)	(37-70)	(19-72)
Sex, n				
Male	8	4	2	2
Female	7	1	3	3
Stage at inclusion (AJCC 7th edition), n				
Stage IIIA	3	1	1	1
Stage IIIB	6	2	2	2
Stage IIIC	5	2	2	1
Stage IIIX	1	0	0	1
Number of metastatic LN, n				
1	8	1	4	3
2-3	3	2	0	1
≥ 4	4	2	1	1
Localization LN dissection, n				
Head/neck	1	1	0	0
Axilla	5	3	2	0
Groin	9	1	3	5
In transit metastases or (micro)satellites, n				
Yes	1	0	1	0
No	14	5	4	5
Extracapsular extension, n				
Yes	7	4	1	2
No	7	1	4	2
Unknown	1	0	0	1

AJCC American Joint Committee on Cancer staging system, *combiDC* combination of mDC and pDC, *DC* dendritic cell, *LN* lymph node, *mDC* myeloid dendritic cell, *pDC* plasmacytoid dendritic cell

Table 2 Treatment-related adverse events (n = 15)

Event*	Any grade (n)	Grade 3 (n)	Grade 4 (n)
Any	15	1	1
Hypocalcemia**	2	0	1
Hypokalemia**	1	1	0
Fatigue	11	0	0
Hypophosphatemia	5	0	0
Paresthesia**	5	0	0
Skin pain (at injection site)	4	0	0
Eosinophil count increased	3	0	0
Hypernatremia	3	0	0
Injection site reaction	3	0	0
ALAT increased	2	0	0
Arthralgia	2	0	0
Blood bilirubin increased	2	0	0
Flu like symptoms	2	0	0
Hematoma (at injection site)	2	0	0
Skin hypopigmentation	2	0	0

*Adverse events of at least grade 3 or grade 1-2 and occurring in more than 1 patient are shown.
**Related to apheresis

Induction of antigen-specific T cell responses and antigen expression in melanoma tissue
After the first cycle of three vaccinations, DTH skin tests were performed to analyze the presence of antigen-specific T cells in SKILs. Tetramer- or dextramer-staining of SKILs of HLA-A2.1 positive patients showed antigen-specific $CD8^+$ T cells in 4 out of 5 (80%) patients tested after the first vaccination cycle (**Supplementary Table 2**). In PBMCs, antigen-specific $CD8^+$ T cells were found in 7 out of 11 (64%) HLA-A2.1, HLA-A1 or HLA-B35 positive patients tested. A lower detection rate of antigen-specific $CD8^+$ T cells in PBMCs compared to SKILs is in line with previous findings and probably due to the accumulation of antigen-specific T cells at the DTH injection site in contrast to a low frequency of those cells circulating in the peripheral blood.[11, 35, 40, 41]

Functionality of T cells was tested by IFNγ production of SKILs cocultured with autologous PBMCs loaded with the relevant antigens. After the first vaccination cycle, IFNγ-producing T cells were found in 9 out of 14 (64%) patients tested. Functional T cells were detected more often in patients vaccinated with pDCs or the combiDCs than in patients treated with mDCs alone, as T cells of 5 out of 5, 4 out of 5 and 0 out of 4 patients per group produced IFNγ, respectively. For patients with functional IFNγ-producing T cells after the first vaccination cycle, median RFS was not reached compared to 17.1 months for patients without functional T cells (p = 0.18) (**Supplementary Figure 3**).

Of the 14 tested patients, 4 patients showed functional T cells against the NY-ESO-1 overlapping peptide pool of whom one (25%) patient showed response against the NY-ESO-1 peptide mix. For MAGE-A3, 2 (33%) of the 6 patients with a functional T cell response against the overlapping peptide pool showed a functional response against the peptide mix as well. The more frequent responses to the overlapping peptide pools substantiate the potential benefit of their addition to the mixes of peptides.

In this study, patients were not selected based on their HLA-type and all patients received DCs loaded with the complete peptide mix. Patients can only potentially respond immunologically to vaccination when the HLA-type of the patient matches the HLA-type of the HLA-binding peptides loaded on the DCs. In addition to HLA-binding peptides, for both MAGE-A3 and NY-ESO-1 overlapping peptide pools were used, which enables potential responses independent of HLA-type. Therefore, every patient could respond to the MAGE-A3 and NY-ESO-1 overlapping peptide pools. In addition, based on their HLA type, all patients could at least respond to one of the HLA-specific peptides used for antigen loading. When all cycles are taken into account, none of the patients showed a T cell response against an antigen they could not potentially respond to. Eight out of 15 (53%) patients could potentially respond to the TAA gp100 and tyrosinase and responses were discovered in 13% and 38% of those patients, respectively (**Figure 2**). Fifty percent of patients that could potentially respond to the CTA MAGE-C2 did show a T cell response. All patients could respond to the MAGE-A3 peptides or peptide pool and in 11 (73%) of them a T cell response was detected. In 8 (73%) of the 11 patients that could respond to NY-ESO-1 peptides, specific T cells were found. All patients could respond to the NY-ESO-1 peptide pool and 53% of them responded.

We studied the expression of the antigens used for antigen loading of the DC product in the primary melanoma, lymph node metastasis and lesion of recurrent disease of vaccinated patients. Representative images are shown in **Supplementary Figure 4**. Gp100 and tyrosinase were expressed in all lesions analyzed (**Figure 3**). Expression of MAGE-C2, MAGE-A and NY-ESO-1 was expressed in a lower number of patients: MAGE-C2 in 36%, MAGE-A in 36% and NY-ESO-1 in 7% of the primary lesions. MAGE-C2 was expressed in 58% of lymph node metastases analyzed, for MAGE-A and NY-ESO-1 this was 42% and 25%, respectively. In 7 (50%) patients from whom tissue was analyzed, the antigen against which a T cell response was detected and the antigen expression on the last resected tumor tissue prior to the start of the experimental adjuvant therapy, matched.

Clinical responses

Nine of 15 patients completed 3 cycles of 3 vaccinations, of whom 7 patients are still free of recurrence. The majority of patients with recurrent disease had stage IIIC melanoma at inclusion. Patient coDC-3 withdrew after two cycles of vaccinations due to personal circumstances but has no recurrent disease. Median RFS for all patients was 19.1 months (**Supplementary figure 5A**). For patients that received mDC vaccination median RFS was 17.1 months. In the pDC and combiDC group median RFS was not reached. Median OS has not been reached at time of data cutoff (**Supplementary figure 5B**). Three patients died from a melanoma-related event, 2 of them received combined mDCs and pDCs and one mDCs alone. Subgroups are too small and heterogeneous to draw conclusions about differences between subgroups, however the combination does not seem to be inferior to vaccination with either subset alone.

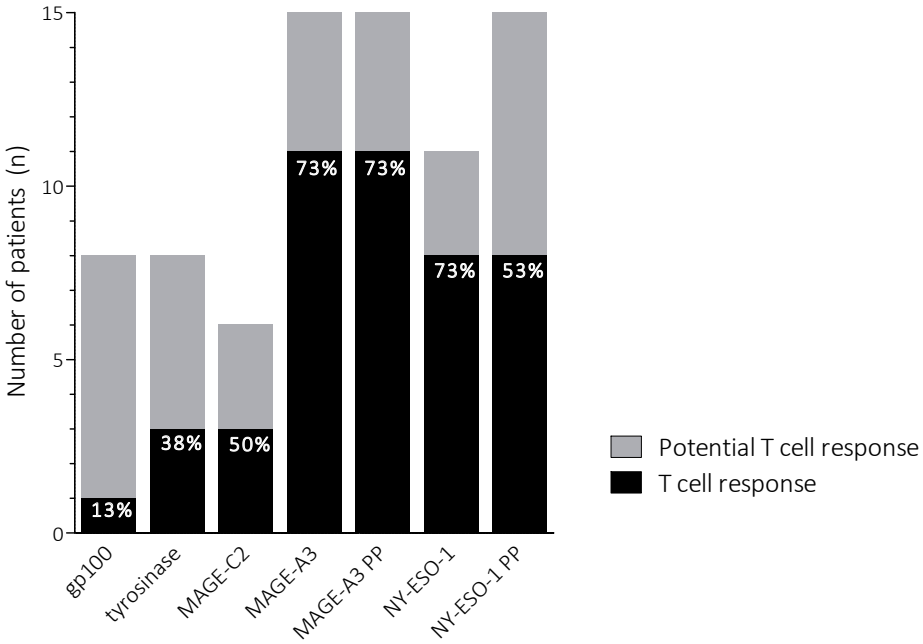
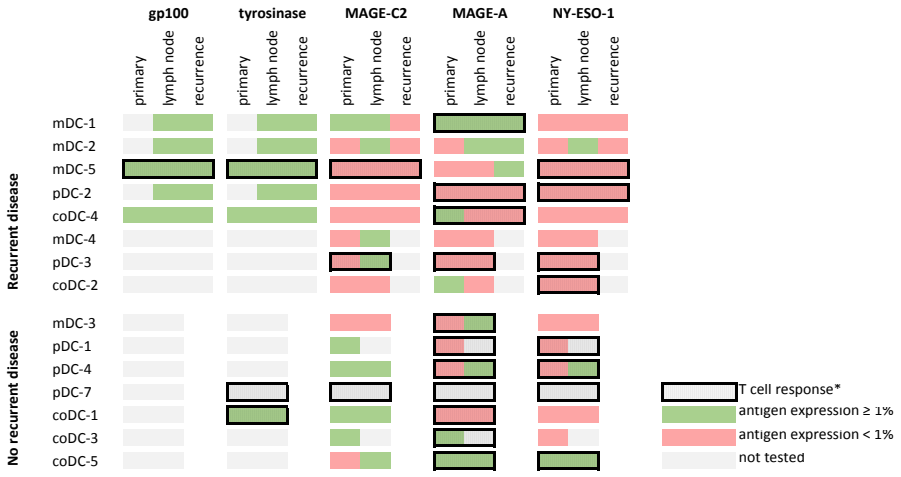


Figure 2 Antigen-specific T cell responses

Total bar height in grey represents the number of patients with a potential immunological response to each of the antigens based on patients' HLA type. The black bar represents the number of patients with a T cell response defined as either antigen-specific CD8+ T cells in skin-test infiltrating lymphocytes (SKILs) or peripheral blood mononuclear cells, or functional T cells in SKILs. *PP* overlapping peptide pools



* T cell response was scored positive when SKILs or PBMCs showed DM+CD8+ T cells or SKILs showed IFNγ-producing T cells

Figure 3 Immunological response and antigen expression

*T cell response was scored positive when antigen-specific $CD8^+$ T cells were present in skin-test infiltrating lymphocytes (SKILs)/ peripheral blood mononuclear cells or IFNγ-producing T cells in SKILs

Discussion

This is the first clinical trial in which melanoma patients were vaccinated with the combination of CD1c+ mDCs and pDCs. Vaccination with CD1c+ mDCs and pDCs alone or combined induced immunological responses. Production of the combined vaccine proved to be feasible. In addition, treatment was well tolerated and the mild toxicity profile is in line with earlier trials investigating DC vaccination.[3, 42] Treatment groups were too small to draw conclusions about differences in immunological or clinical response between the three groups. However, combination therapy does not seem to be inferior to vaccination with CD1c+ mDCs or pDCs alone.

In this trial CD1c+ mDCs were predepleted for CD14+ cells and both CD1c+ mDCs and pDCs were maturated by stimulation with pR, a TLR8/9 stimulus. Production of nDC vaccines meeting predefined release criteria for purity, maturation and viability was achieved for all patients in all groups. PR-matured pDCs produced high amounts of IFNα, which is a common hallmark of activated pDCs and important for their function in enhancing the cellular response and stimulation of cells of the innate immune system important for an anti-tumor response.[43, 44] PR-matured CD1c+ mDCs produced IL-12, important to turn naive CD4+ and CD8+ T cells into T-helper 1 and cytotoxic T cells, respectively.[45] In addition, costimulatory molecules were highly expressed on both subsets after pR maturation, which is in line with our previous findings.[19]

In this trial, mDCs and pDCs were cultured separately and pooled before injection. Coculturing might further improve the nDC vaccine as this enables cross-activation between mDCs and pDCs during maturation, possibly leading to a superior maturation state of DCs, as previously shown.[46] It has been shown previously that when mDCs and pDCs are matured in a coculture, cytokine production and expression of costimulatory molecules is higher and the activation of other immune cells that could play an important role in the anti-tumor response, such as natural killer cells and plasma cells, is stronger.[24, 44, 46, 47] Furthermore, nDC vaccination could be further improved by the addition of the even less frequent CD141+ (BDCA-3) subset of mDCs. These cells play an important role in cross presentation of necrotic cell-derived antigens and activation of CD8+ T cells.[48-50] Recently, it became possible to select these scarce CD141+ mDCs from the blood by magnetic separation, however this method is not yet available for clinical application.[51] All three subsets of nDCs have distinct phenotypes and mechanisms of influencing the immune response, probably making their function complementary. Hence, and considering the excellent capacity of CD141+ DCs to cross-present tumor cell-derived antigens, DC vaccination with a combination of all three subsets could induce even more potent anti-tumor responses as compared to either of the single DC subsets.[52]

We expanded the pool of antigens of the two TAA gp100 and tyrosinase used in previous trials, with the three CTA MAGE-C2, MAGE-A3 and NY-ESO-1 which are present frequently in melanoma.[21] Immunological responses against each of the antigens used were observed. The expression of CTA in melanoma tissue was lower compared to TAA. As such, analysis of the antigen expression prior to the start of study could further improve the selection of

antigens used. As was done in this trial, it is important to take the frequency of HLA types into account when peptides binding certain HLA-types are used for antigen loading. This can be circumvented by the use of overlapping peptide pools as was done in this trial, which increased the immunological responses against these peptides. Overlapping peptide pools are therefore of interest for future trials in addition to the use of individual peptides.

Immunological responses were detected in the majority of patients. After the first vaccination cycle antigen-specific $CD8^+$ T cells in SKILs were found in 80% and functional T cells in 64% of tested patients treated with adjuvant nDC vaccination. These numbers are at least comparable to the amount of antigen-specific $CD8^+$ and functional T cells after adjuvant moDC vaccination of stage III melanoma patients we reported earlier and higher than was reported for $CD1c^+$ mDC or pDC vaccination in stage IV melanoma.[10, 11, 29] Drawing conclusions about a possible difference between nDC and moDC vaccination is difficult because clinical characteristics of stage III patients are not homogeneous and more antigens were used in the here described trial. The number of patients with functional T cells after combined $CD1c^+$ mDC and pDC vaccination was as high as for pDC vaccination alone, and might be superior compared to $CD1c^+$ mDC vaccination alone. Although groups are small, the combination does not seem to be inferior in eliciting immunological response, compared to the single subsets alone and allows application in a larger patient cohort.

In one patient (mDC-5) with a T cell response against both gp100 and tyrosinase, one patient (coDC-1) with a T cell response against tyrosinase and one patient (mDC-1) with a T cell response against MAGE-A3, the respective antigens were present in the tumor tissue prior to the start of study and in mDC-5 and mDC-1 remained present in the recurrent lesion. The continuation of antigen expression despite an antigen-specific T cell response indicates incomplete killing of the antigen-expressing tumor cells. This might be due to immune suppressive factors, suppressing the cytotoxicity of T cells. Escape mechanisms that might be present are for example immunosuppressive immune cells such as myeloid-derived suppressor cells and regulatory T cells or the upregulation of inhibitory immune checkpoints such as CTLA-4 and PD-L1.[53] The addition of therapy interfering with these immunosuppressive mechanisms inhibiting the anti-tumor effect of DC vaccination is therefore of interest for future research. Combination of DC vaccination with mAbs against the checkpoint molecule CTLA-4, which inhibits activation of T cells, already showed promising clinical responses when combined with DC vaccination.[54, 55] MAbs interfering with the PD-1/PD-L1 pathway inhibit T cell exhaustion in the TME and could therefore also enhance the anti-tumor effect of T cells induced by DC vaccination. Due to their superior survival benefit and toxicity profile compared to anti-CTLA mAbs it is a logical next step to combine anti-PD-1 mAbs and DC vaccination in future research investigating combination therapy.[56, 57]

We included stage III melanoma patients, a heterogeneous group, of which most were high-risk patients (stage IIIB or IIIC). Because of the high risk of relapse, multiple phase III trials have been conducted investigating ICI and targeted therapy. Results of these trials led to the

approval of the ICI nivolumab and pembrolizumab (both anti-PD-1 mAbs) based on improved RFS and targeted therapy with combined dabrafenib and trametinib (BRAF and MEK inhibitor) for BRAF V600-mutated melanoma based on OS benefit by both the European Medicines Agency (EMA) and Food and Drug Administration (FDA).[32-34] In addition, the FDA approved adjuvant use of the ICI ipilimumab (anti-CTLA-4 mAbs).[58] However, toxicity of these adjuvant therapies is high as 14-46% of patients encountered treatment-related grade 3-4 events.[32-34, 58] The mild toxicity profile of adjuvant DC vaccination is favorable compared to these registered treatment options, as only grade 1-2 adverse events occurred and these mainly consisted of fatigue.

As previously shown, nDC vaccination does not withhold a clinically relevant improvement of the HRQoL, probably due to recovery from RLND, during adjuvant therapy.[39] This finding is supported by HRQoL data of DC vaccination in other malignancies.[59, 60] HRQoL of patients treated with adjuvant nivolumab or dabrafenib/trametinib remained unchanged during the trial.[32, 61] However, despite the expected recovery from RLND, a non-clinically relevant decline in HRQoL during adjuvant treatment with ipilimumab and pembrolizumab was found.[62, 63] In the adjuvant setting HRQoL is an important aspect, as half of the stage III patients will not endure a relapse without adjuvant therapy, but are exposed to the potentially severe toxicity. In addition, melanoma patients are relatively young and the majority is of working-age, making the impact on HRQoL even more important. Of course, impact on survival is critical in the choice of therapy. The survival benefit of adjuvant nDC vaccination is currently investigated in our placebo-controlled randomized controlled trial (NCT02993315) in which patients receive combined CD1c+ mDCs and pDCs. In addition, the OS benefit of the phase III trials investigating adjuvant nivolumab and pembrolizumab are awaited.

In conclusion, production of a vaccine with combined CD1c+ mDCs and pDCs is feasible. Adjuvant treatment of stage III melanoma patients with both nDC subsets simultaneously was well tolerated with only mild grade 1-2 adverse events. DC-based immunotherapy was capable of inducing immunological response and combined CD1c+ mDC and pDC vaccination was not inferior to vaccination with each of the single subsets alone. This allowed further exploration of the clinical response to combined DC vaccination in a placebo-controlled clinical trial in which patients were enrolled prior to the approval of the currently registered adjuvant therapies. Results are expected at the beginning of 2021.

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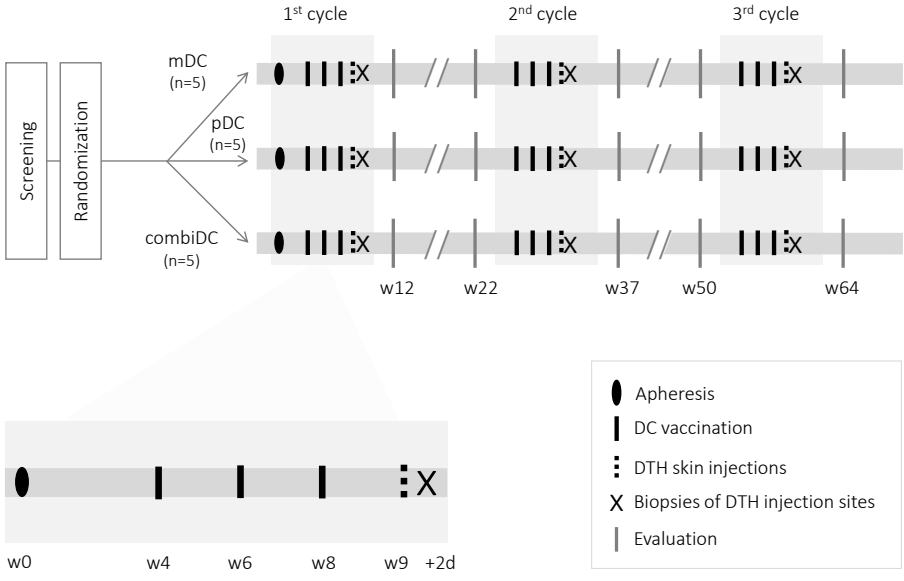
Supplementary Material

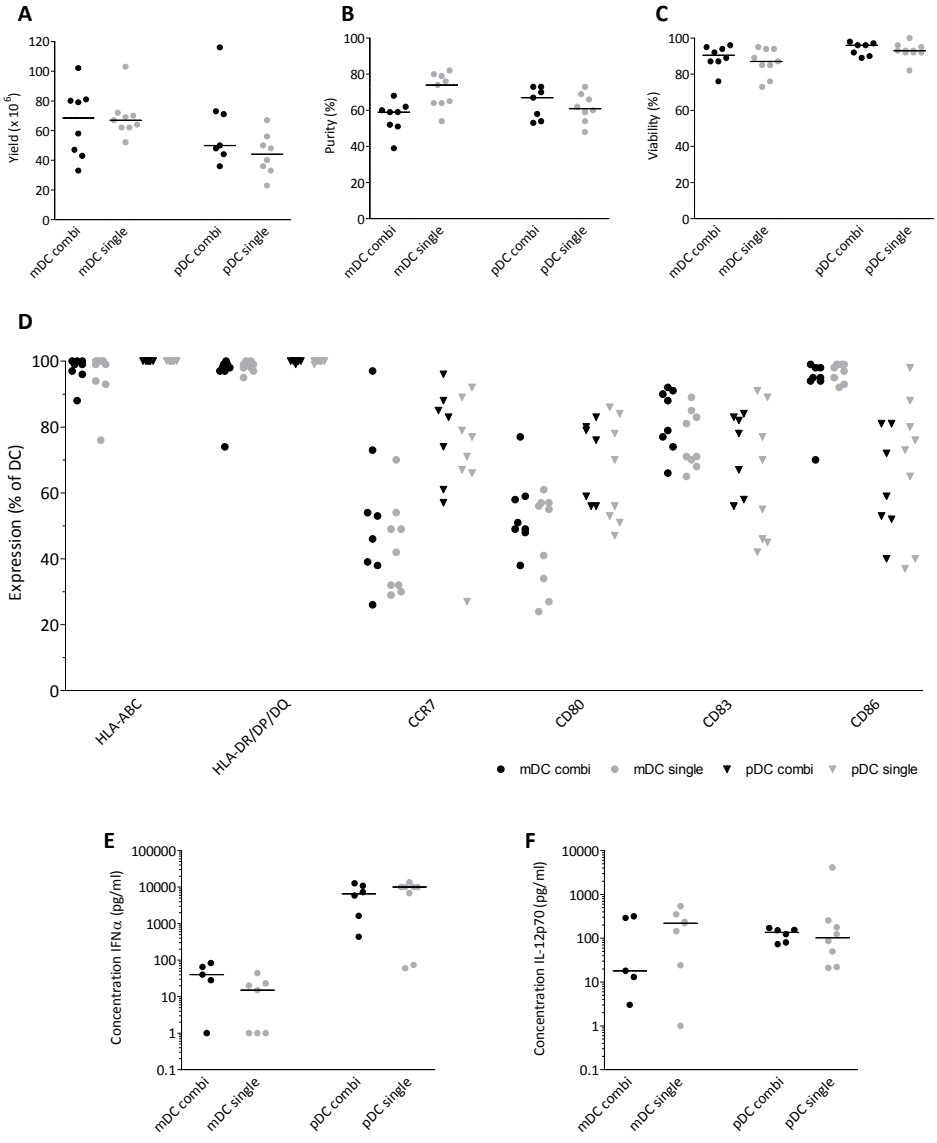


Supplementary Figure 1 Study schedule

After successful screening, patients were randomized 1:1:1 to receive mDC or pDC vaccination or the combination of both (combiDC). An apheresis was performed to collect peripheral blood mononuclear cells. Treatment phase consisted of 3 cycles of 3 biweekly intranodal DC vaccinations. If necessary the apheresis was repeated prior to the start of the second and/or third cycle. Evaluation consisted of medical history, physical examination and imaging when indicated. *d* day, *DC* dendritic cell, *DTH* delayed-type hypersensitivity, *IN* intranodal, *mDC* myeloid DC, *pDC* plasmacytoid DC, *w* week

Supplementary Table 1 Peptides used for vaccine preparation

Antigen	Peptide	Position	HLA
Gp100	KTWGQYWQV	154-162	A2.1
	YLEPGPVTA	280-288	A2.1
	WNRQLYPEWTEAQRLD	44-59	DR4
Tyrosinase	YMDGTMSQV	369-377	A2.1
	DYSYLQDSDPDSFQD	448-462	DR4
MAGE-C2	ALKDVEERV	336-344	A2.1
	SESIKKKVL	307-315	B44
MAGE-A3	KVAELVHFL	112-120	A2.1
	EVDPIGHLY	168-176	A1
			B35
	EGDCAPEEK	212-220	Cw7
	KKLLTQHFVQENYLEY	243-258	DP4
			DQ6
	PepTivator® peptide pool	Overlapping peptides	Multiple
NY-ESO-1	SLLMWITQC	157-165	A2.1
	ASGPGGGAPR	53-62	A3
			A31
	MPFATPMEA	94-102	B35
	PepTivator® peptide pool	Overlapping peptides	Multiple



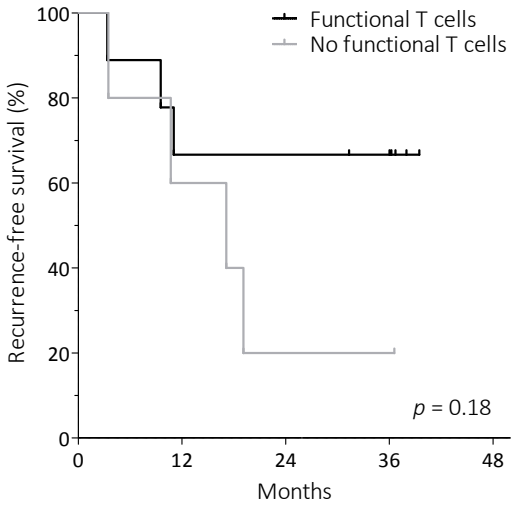
Supplementary Figure 2 Dendritic cell product characteristics

(**A**) Yield, (**B**) purity and (**C**) viability of the dendritic cell (DC) product. (**D**) Phenotype of the DC product after 3 hours of maturation. In one patient in the pDC group, CD80 expression was 47% after 3 hours, but met release criteria after 6 hours. Production of (**E**) IFNα and (**F**) IL-12p70 after 6 hours of maturation. *mDC* myeloid DC, *pDC* plasmacytoid DC

Supplementary Table 2 Immunological and clinical response after the first cycle of vaccines

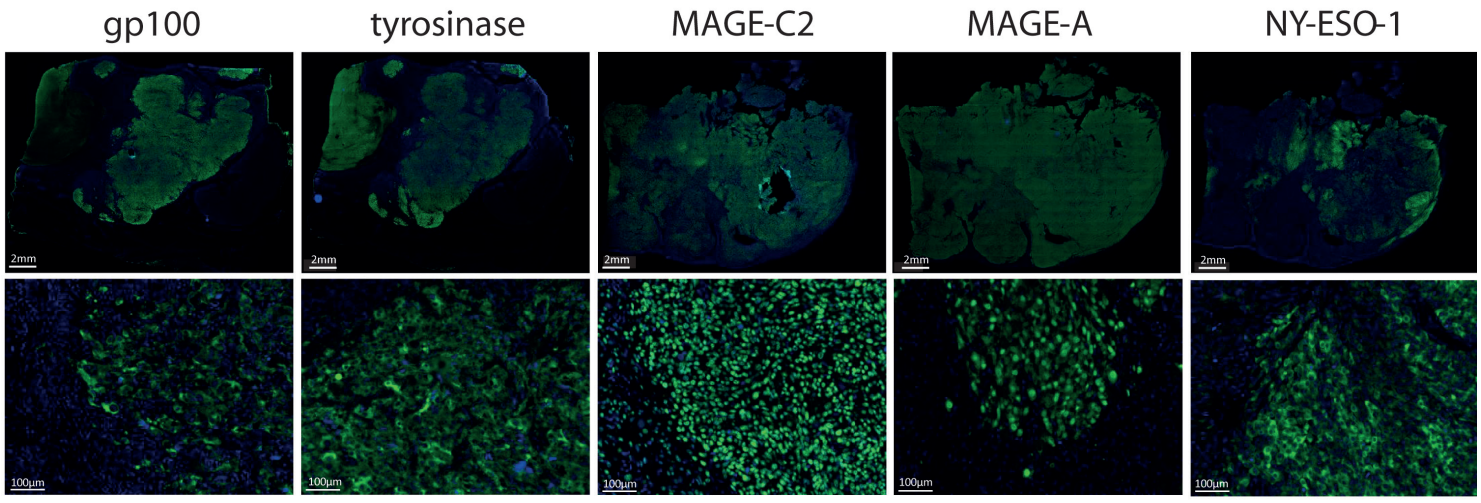
Study code	Stage at inclusion (AJCC 7th edition)	Number of vaccines received	Tumor antigen specific T cell response by DM staining			IFNγ-producing SKILs	RFS (months)	Current status
			HLA-A2.1	PBMCs	SKILs			
mDC-1	IIIC	9	-	nt	nt	nt	13.4	MRD
mDC-2	IIIB	9	-	nt	nt	-	17.1	Alive with disease
mDC-3	IIIB	9	-	+	nt	-	36.6+	NED
mDC-4	IIIC	3	-	nt	nt	-	3.5	Alive with disease
mDC-5	IIIA	9	+	+	+	-	19.1	Alive with disease
pDC-1	IIIA	9	-	-	nt	+	36.0+	NED
pDC-2	IIIC	6	-	+	nt	+	11.0	Alive with disease
pDC-3	IIIC	6	+	+	+	+	9.5	Alive with disease
pDC-4	IIIB	9	-	+	nt	+	36.2+	NED
pDC-7	IIIB	9	+	+	+	+	31.3+	NED
coDC-1	IIIB	9	+	+	+	+	39.5+	NED
coDC-2	IIIC	3	-	-	nt	+	3.4	MRD
coDC-3	IIIB	6*	-	nt	nt	+	38.0+	NED
coDC-4	IIIA	6	-	-	nt	-	10.7	MRD
coDC-5	IIIX	9	+	-	-	+	36.7+	NED

*Withdrew after 2 cycles due to personal circumstances. *AJCC* American Joint Committee on Cancer staging system *IFN* interferon, MRD melanoma-related death, *NED* no evidence of disease, *nt* not tested, *PBMCs* peripheral blood mononuclear cells, *RFS* recurrence-free survival, *SKILs* skin-test infiltrating lymphocytes

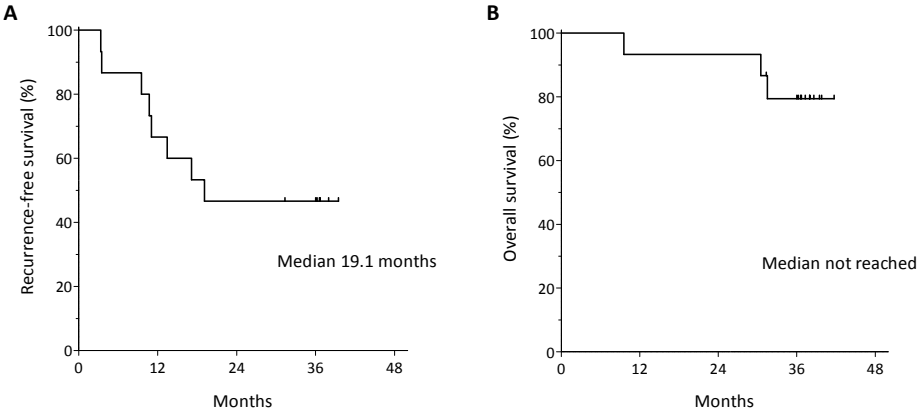


Supplementary Figure 3 Survival curve

Kaplan-Meier estimate for recurrence-free survival in patients showing functional, interferon-γ secreting, T cells after the first cycle, compared to patients without functional T cells



Supplementary Figure 4 Immunohistochemical staining of the different antigens
Representative images of antigen expression staining depicted in green and DAPI staining in blue on slides of whole tissue sections (**top panel**) and original magnification 20x (**bottom panel**)



Supplementary Figure 5 Survival curves

Kaplan-Meier estimates of (**A**) recurrence-free survival and (**B**) overall survival.

4

Health-related quality of life analysis in stage III melanoma patients treated with adjuvant dendritic cell therapy

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Abstract

Background. Health-related quality of life (HRQoL) is an important issue in the rapidly evolving field of adjuvant treatment for stage III melanoma. Dendritic cell vaccination is one of the adjuvant forms of therapy currently investigated.

Methods. We enrolled adults with stage III melanoma to receive adjuvant dendritic cell vaccination after a complete radical lymph node dissection. HRQoL assessment was one of the secondary endpoints of this trial and investigated with the EORTC-QLQ-C30 questionnaire at baseline and week 26.

Results. Fifteen patients with a median age of 50 years were included in the study, with 12 evaluable patients on study at time of the second questionnaire. Global health status and role functioning improved clinically relevant with a mean difference of 15 (p = 0.010) and 26 points (p = 0.005), respectively.

Discussion. Despite the small number of patients, we found a clinically relevant improved global health status. Besides, compared to the other investigated therapies, toxicity of dendritic cell vaccination is low, which supports our finding.

Conclusion. This is the first description of HRQoL in melanoma patients receiving dendritic cell vaccination. We show the expected improvement in global health status after surgical treatment of stage III melanoma. Thus, adjuvant dendritic cell vaccination does not seem to hamper this improvement, as shown in our small explorative study.

Background

The incidence of melanoma is increasing and in 2012 over 20,000 persons in Europe died due to melanoma.[1, 2] Patients with regional lymph node metastasis, but without distant metastasis, are considered to have stage III disease. When operable, stage III melanoma is treated surgically and with curative intent. Until the recent publication of the MSLT-II trial, this included a radical lymph node dissection (RLND) in all patients.[3] Unfortunately, even after complete resection, stage III melanoma bears a high risk of recurrence with a 5-year overall survival (OS) rate of 78%, 59%, and 40% in the substages IIIA, IIIB and IIIC, respectively.[4] Due to this high recurrence rate adjuvant treatment is warranted.

Current adjuvant treatment consists of radiotherapy to reduce the risk of local relapse in high-risk melanoma, without an effect on OS.[5] Clinical trials investigating adjuvant use of immune checkpoint inhibitors (ICI) and targeted therapies have been conducted. Both treatment with ipilimumab (anti-CTLA-4 antibody), and combined dabrafenib (BRAF inhibitor) and trametinib (MEK inhibitor) have shown a significant improved OS compared to placebo.[6, 7] Nivolumab and pembrolizumab (both anti-PD-1 antibodies) were compared with ipilimumab and placebo, respectively. Both have shown a significantly improved recurrence-free survival (RFS) compared to the control arm and data on OS are awaited.[8, 9] At time of writing, ipilimumab, nivolumab, pembrolizumab as well as combined dabrafenib and trametinib are approved as adjuvant treatment by the Food and Drug Administration (FDA). The European Medicine Agency (EMA) approved use of adjuvant nivolumab and combined dabrafenib and trametinib. The Committee for Medicinal Products for Human Use (CHMP) gave a positive advice to the EMA about adjuvant use of pembrolizumab.

All these therapies have significant toxicity. Grade 3 or 4 adverse events occurred in 41.4% or 54.1% with dabrafenib/trametinib and ipilimumab, respectively.[6, 7] Nivolumab and pembrolizumab have less significant toxicity with 14.4% and 14.7% treatment-related grade 3, 4 or 5 adverse events.[8, 9] One patient receiving anti-PD-1 treatment died, caused by pembrolizumab-induced myositis.[8] In contrast to the toxicity, assessment of the mean global health score in patients receiving adjuvant ipilimumab or nivolumab showed no clinically meaningful differences compared to baseline.[9]

Dendritic cell (DC) vaccination is another form of immunotherapy, which seems to be more powerful in the adjuvant than metastatic setting, possibly due to the lower amount of tumor, hence less tumor-induced immune suppression.[10] This is supported by the improvement in OS we observed retrospectively in stage III melanoma patients vaccinated with DC, compared to matched controls receiving standard of care consisting of follow-up.[11]

Adjuvant treatment options are emerging and survival benefits will be compared. Besides survival, it is important to take HRQoL into account since approximately half of stage III melanoma patients will not relapse without adjuvant treatment, but are exposed to the potential side effects.[4] In addition, a large number of patients are of working-age with a

median age of 59 years (range 5-98) and therefore, normal life expectancy in this population is high.[12] In sharp contrast to ICI, severe toxicity is rare with DC vaccination and an advantage of this form of cellular immunotherapy, thus we hypothesized HRQoL is better with this form of therapy.[13, 14]

To our knowledge, HRQoL in melanoma patients receiving DC-based therapy is not yet reported. Results from trials with DC vaccination in other forms of cancer do not show a negative impact on HRQoL. For example, in a trial with metastatic renal cell carcinoma patients, DC vaccination did not hamper HRQoL.[15] In another trial investigating patients with disseminated colorectal carcinoma receiving DC vaccination, HRQoL remained high and stable on most of the scales, except for general health perception and vitality.[16] Our study aims to investigate the HRQoL of stage III melanoma patients treated with adjuvant DC vaccination in our explorative study.

Methods

Study design

Prospective study, investigating HRQoL during adjuvant DC vaccination in patients with stage III melanoma, conducted at the Radboud University Nijmegen Medical Center. This study is registered as NCT02574377 at ClinicalTrials.gov. The protocol has been approved by the national Review Board (Central Committee on Research involving Human Subjects), and is in concordance with the Declaration of Helsinki and Good Clinical Practice. Written informed consent was obtained from all individual participants.

Patients

Eligibility criteria included histologically documented stage III melanoma, a complete RLND within 12 weeks before start of study and a WHO performance score of 0 or 1. Patients with serious active infection, immunosuppressive conditions, or adjuvant radiotherapy were excluded. Immunological response was the primary endpoint of this study (manuscript in preparation). HRQoL analysis was one of the secondary endpoints.

Methods

Patients underwent an apheresis to acquire autologous mononuclear cells. Naturally occurring DCs were collected from the apheresis product using the CliniMACS Prodigy® and GMPgrade magnetic bead-coupled antibodies (both Miltenyi Biotec, Bergisch Gladbach, Germany), matured with protamine-mRNA, and loaded with peptides of gp100, tyrosinase, MAGE-A3, MAGE-C2 and NY-ESO1.[17] After quality assessments, on average 3.6×10^6 DCs were injected in a clinically tumor-free lymph node under ultrasound guidance. One cycle consisted of 3 biweekly intranodal injections. In the absence of disease recurrence, 2 maintenance cycles of 3 injections each were given, with a 6-month interval between cycles as shown in **Figure 1**. Toxicity assessment was performed before every vaccination, 1 week after a cycle as well as 3-5 weeks before and after the start of each cycle. Toxicity was scored according to the Common Terminology Criteria for Adverse Events, version 4.0.

HRQoL assessment

The European Organization for Research and Treatment of Cancer Quality of Life, Quality of Life core Questionnaire C30 (EORTC QLQ-C30) version 3.0 was used to assess HRQoL.[18] This 30-item questionnaire contains five functional scales on physical, role, emotional, cognitive, and social functioning, a global health status scale, three symptom scales on fatigue, nausea and vomiting and pain, and six single items on dyspnea, insomnia, appetite loss, diarrhea, constipation and financial difficulties. The questions were framed as 'during the past week…'. Response scales included: 'Not at all', 'A bit', 'Quite a bit', and 'Very much', except for the global health status scale, which ranges from 'Very poor' to 'Excellent'. Scores

were linearly transformed to a 0 – 100 scale.[19] For the EORTC QLQ-C30, a higher score on the functioning-related domains represents a better quality of life and functioning. A higher score in one of the symptom-related domains represents a worse level of symptoms. Administration of the HRQoL questionnaires followed the clinical assessment schedule of the study. Therefore questionnaires were completed at baseline before apheresis, at the start of each subsequent cycle, as shown in **Figure 1**, and once a year thereafter for up to 5 years. Due to the small number of patients and high recurrence rate in this patient category, we report the HRQoL during the first cycle. The Cronbach's alpha-coefficient of our data is 0.86, which suggests a good internal consistency.

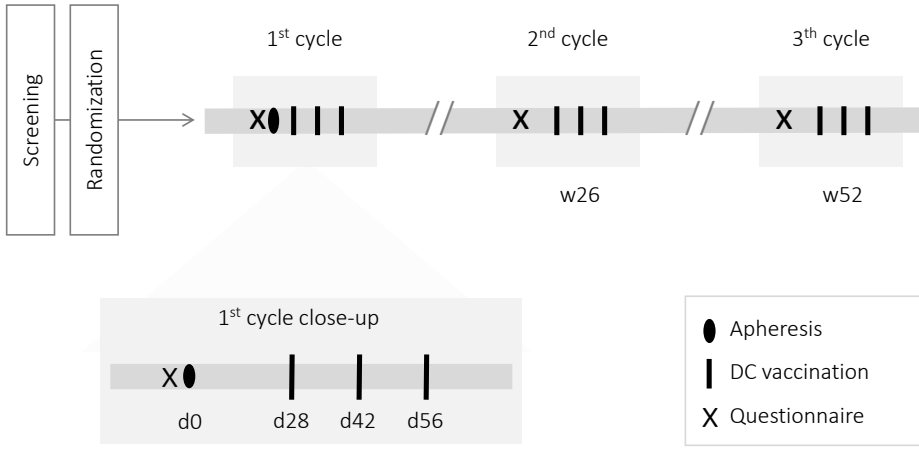

Figure 1 Study schedule

Statistical analysis

To describe change in HRQoL during study participation, mean scale scores between patients at baseline and after 26 weeks, at the start of cycle 2, were compared using paired t-tests. All statistical analyses were conducted using SPSS Statistics version 22 (IBM Inc., Armonk, NY, USA). Figures were created with GraphPad Prism, version 5.03 (GraphPad Software, Inc., La Jolla, CA, USA). Besides reporting the statistical significant differences, considered as p values < 0.05, more importantly we investigated clinically relevant differences. According to the work of Osoba et al. and King, a mean change of 10 points on a scale score was defined as clinically relevant.[20, 21] Missing items from multi-item scales of the EORTC QLQ-C30 were mean-imputed if at least half of the items from a scale were completed, according to the EORTC QLQ-C30 scoring guidelines.[22]

Results

Patient characteristics

Fifteen patients were included in the study between October 2015 and August 2016. Two patients dropped out due to recurrent disease before week 26 and one patient was excluded from analysis due to completion of the baseline questionnaire after apheresis. The remaining 12 patients who completed the questionnaire at baseline before apheresis and at week 26, were analyzed. Completion compliance was 100% for both time points and only one item was missing. Baseline characteristics are shown in **Table 1**. Median age of patients was 50 years (range 19-72 years). Seven patients were male (58%) and five were female (42%). All of the AJCC 2009[4] stage III categories were represented. Most patients underwent an inguinal (67%) or axillary (25%) RLND and one patient underwent a neck RLND.

Table 1 Baseline characteristics (n = 12)

Characteristic	
Age	
Median – range (year))	50 (19-72)
Sex, n (%)	
Male	7 (58)
Female	5 (42)
WHO performance score, n (%)	
0	2 (17)
1	10 (83)
Stage at inclusion, n (%)	
IIIA	3 (25)
IIIB	6 (50)
IIIC	2 (17)
IIIx	1 (8)
Site of RLND, n (%)	
Head and neck	1 (8)
Axilla	3 (25)
Groin	8 (67)

RLND radical lymph node dissection

Adverse events

Adverse events, considered as possibly, probably or definitely related to study treatment, until week 26 are presented in **Table 2**. Patients may have had more than one adverse event. All adverse events were grade 1 or grade 2, except in one patient. The only grade 3 and 4 events were hypokalemia and hypocalcemia, respectively, both caused by apheresis. These disturbances were corrected with suppletion, and besides transient paresthesia, without clinical consequences.

Table 2 Drug-related adverse events until week 26 (n = 12)

Adverse event	Any grade n (%)	Grade 3 n (%)	Grade 4 n (%)
Any adverse event	11 (85)	1 (8)	1 (8)
Hypocalcemia	0	0	1 (8)
Hypokalemia	0	1 (8)	0
Fatigue	8 (67)	0	0
Flu like symptoms	2 (17)	0	0
Injection site reaction	2 (17)	0	0
Chills	1 (8)	0	0
Dizziness	1 (8)	0	0
Dry eye	1 (8)	0	0
Eosinophilia	1 (8)	0	0
GGT increased	1 (8)	0	0
Headache	1 (8)	0	0
Hypophosphatemia	1 (8)	0	0
Monocytosis	1 (8)	0	0
Paresthesia	1 (8)	0	0
Skin irritability	1 (8)	0	0
Urea elevated	1 (8)	0	0

Adverse events considered as possibly, probably or definitely related to the study drug

Functioning-related domains of HRQoL

Except for cognitive functioning, all functioning-related domains showed an improvement over time. For role functioning this improvement was clinically relevant and significant. Mean scales scores of each of the scales of the EORTC QLQ-C30 questionnaire both at baseline and week 26 are presented in **Figure 2**. Mean differences and clinical relevance are shown in **Table 3**. Physical and emotional functioning improved neither significant nor clinically relevant. Patients presented a clinically relevant and significant increase in role functioning 26 weeks after vaccination (mean difference 26 points, $p = 0.005$). Social functioning showed

a clinically relevant improvement with a mean difference of 16 points, but this difference was not significant (p = 0.082). Cognitive functioning was high at baseline with a mean score of 92, and remained high at week 26 with a mean difference of 0 points (p = 1.000).

Global health status

The global health status scale showed a clinically relevant and significant increase of 15 points (p = 0.010). This indicates an improved global health status over time while receiving adjuvant DC-based therapy.

Symptom-related domains of HRQoL

Pain decreased significantly and clinically relevant with a mean difference of − 19 points (p = 0.019). Besides, financial difficulties decreased significantly and clinically relevant as well, with a mean difference of − 28 points (p = 0.017). None of the other symptom-related domains changed clinically relevant or significant.

Follow-up

At week 52 and week 78, 8 and 6 patients completed the questionnaire, respectively. None of the domains showed a clinically relevant and significant change during these follow-up visits.

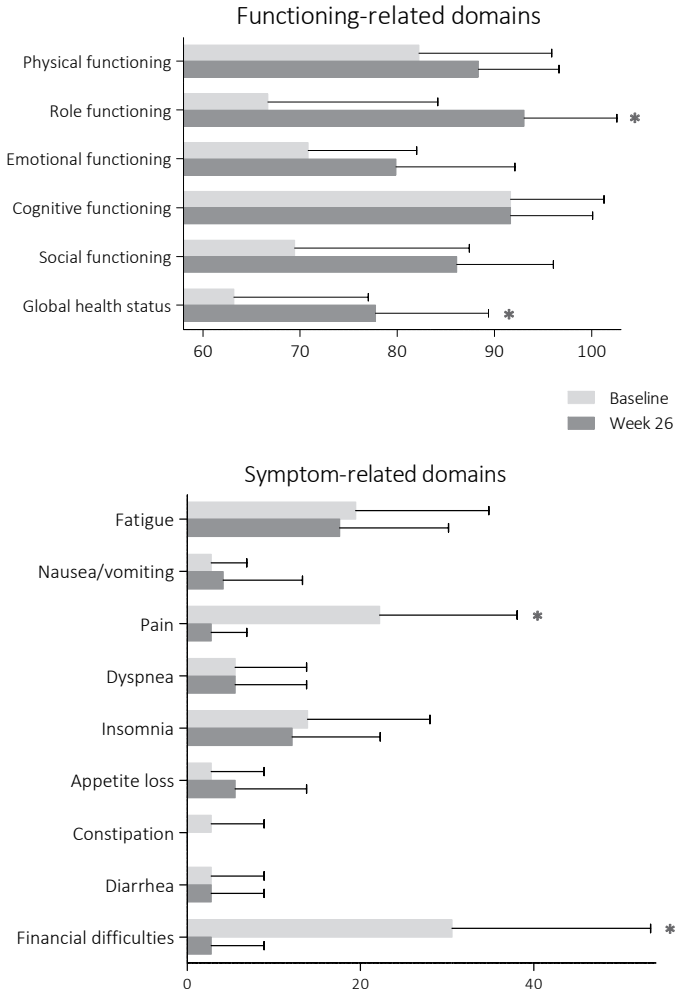

Figure 2 Mean scale scores (0-100) with 95% confidence interval on functioning and symptom-related domains of each of the domains of the EORTC-QLQ-C30 questionnaire at baseline and 26 weeks of treatment.

*Difference is both significant and clinically relevant

Table 3 Mean scores of HRQoL according to the different points in time

	Baseline Score (SD)	Week 26 Score (SD)	Mean difference Points (SD)	*p*-value	CID
QLQ-C30 functioning-related domains					
Physical functioning	82 (22)	88 (13)	6 (14)	0.152	No
Role functioning	67 (28)	93 (15)	26 (26)	0.005	Yes
Emotional functioning	71 (18)	80 (19)	9 (19)	0.127	No
Cognitive functioning	92 (15)	92 (13)	0 (17)	1.000	No
Social functioning	69 (28)	86 (16)	16 (30)	0.082	Yes
Global health status	63 (21)	78 (18)	15 (16)	0.010	Yes
QLQ-C30 symptom-related domains					
Fatigue	19 (24)	18 (20)	-2 (15)	0.674	No
Nausea/vomiting	3 (6)	4 (14)	1 (11)	0.674	No
Pain	22 (25)	3 (6)	-19 (24)	0.019	Yes
Dyspnea	6 (13)	6 (13)	0 (14)	1.000	No
Insomnia	14 (22)	12 (17)	2 (21)	0.776	No
Appetite loss	3 (10)	6 (13)	3 (17)	0.586	No
Constipation	3 (10)	0 (0)	-3 (10)	0.339	No
Diarrhea	3 (10)	3 (10)	0 (14)	1.000	No
Financial difficulties	31 (36)	3 (10)	-28 (34)	0.017	Yes

Mean HRQoL scores range from 0 to 100. A higher score on a functioning-related domain indicates better functioning, whereas a higher score on a symptom-related domain indicates more complaints.
CID clinically important difference, *HRQoL* health-related quality of life, *SD* standard deviation

Discussion

In our study, global health status of patients receiving adjuvant DC vaccination improved clinically relevant over time. In addition, role functioning, pain and financial difficulties showed a clinically relevant improvement. Although preliminary, this is promising data when compared to thus far available HRQoL data of ICI. After initial treatment, an increase in HRQoL was expected due to recovery from diagnosis and surgical treatment. In phase III trials investigating HRQoL of stage III melanoma patients while receiving adjuvant therapy, the observation or placebo arm indeed showed an increase in global health status over time, although not clinically relevant.[5, 23-25] We hypothesize that recovery from RLND is not hampered by the administration of DC vaccination.

Clinical implications

Other trials in the same study population do not report an improvement in global health status. The EORTC 18071 trial investigating adjuvant ipilimumab shows a decline of 4.2 points in global health status at week 24 compared to baseline.[23] Lorigan and Green explain this contradiction between the large number of adverse events with ipilimumab and the relatively good HRQoL by the possibility of missed symptom-related HRQoL reduction caused by timing of the questionnaire. The HRQoL was assessed at week 24, and the last induction vaccination was administered in the ninth week. Symptoms could have been missed since the median time to onset of adverse events is 4-12 weeks and the median time to resolution is 4-8 weeks. In addition, the possible perception of patients having adverse events as a positive reassurance they have been randomized to the treatment arm could have had an impact on the reported HRQoL.[26] The Checkmate-238 trial comparing adjuvant ipilimumab to nivolumab reports data of the global health status of included patients that does not differ clinically relevant from baseline.[9] At time of writing, HRQoL data of the trial investigating combined dabrafenib and trametinib as well as the trial investigating pembrolizumab were not reported.

Besides global health status, financial difficulties improved clinically relevant and significant in our study. At baseline some patients reported difficulties with their financial situation due to their physical situation or medical treatment. In all but one of our patients, these difficulties have disappeared at week 26. We assume this is due to patients being able to work while receiving DC therapy, after recovery from RLND. Being able to work and participate in society is an important aspect of the quality of life for patients of working age with (cured) cancer.[27] The ability to work during treatment is also important for the cost-effectiveness of a treatment. Comparison of financial difficulties with other trials was not possible since the outcomes of this scale were not mentioned separately.[9, 23-25, 28]

Study limitations

The major limitation of our study is the small number of patients. Nevertheless, we found a clinically relevant improvement of the global health status. Besides, compared to the other investigated therapies toxicity of DC vaccination is low, which supports our finding. Another limitation is the lack of a control arm. Participating in a therapeutic trial instead of a wait-and-see policy could have a positive impact on the HRQoL. This bias could be avoided by inclusion of a placebo arm. Currently, we are investigating the HRQoL in our ongoing phase III trial, as further research is needed to confirm our findings.

Conclusion

This explorative study shows promising preliminary results with a clinically relevant improvement of the global health status. As further research on survival benefit, HRQoL and cost-effectiveness is required, we currently investigate these outcomes in our ongoing placebo-controlled phase III trial investigating adjuvant DC vaccination in stage IIIB and IIIC melanoma patients (NCT02993315). In this study, HRQoL is evaluated using the EORTC-QLQ-C30, the EuroQol Five Dimensions Health Questionnaire, and the Functional Assessment of Cancer Therapy - Melanoma questionnaire and cost-effectiveness using the Medical Consumption Questionnaire and the Short Health and Labour Questionnaire.

Funding

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Acknowledgment

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5

Early recurrence in completely resected stage IIIB and IIIC melanoma patients warrants restaging prior to adjuvant therapy

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Abstract

Purpose. To evaluate the results of restaging completely resected stage IIIB/C melanoma prior to start of adjuvant therapy.

Patients and methods. One hundred twenty patients with stage IIIB or IIIC (AJCC 2009) melanoma who underwent complete surgical resection were screened for inclusion in our trial investigating adjuvant dendritic cell therapy (NCT02993315). All patients underwent imaging to exclude local relapse or metastasis before entering the trial. The frequency of recurrent disease within 12 weeks after resection and the method of detection were investigated.

Results. Sixty-nine (58%) stage IIIB and 51 (43%) stage IIIC melanoma patients were screened. Median age was 54 (range 27-79) years. Twenty-two (18%) of 120 patients with completely resected stage IIIB/C melanoma had evidence of early recurrent disease, despite exclusion thereof by prior imaging. Median interval between resection and detection of relapse was 7.4 (range 4.3-10.7) weeks. Recurrence was asymptomatic in 17 (77%) patients, but metastasis was noticed by the patient or physician in 5 (23%). Eight patients with local relapse received local treatment with curative intent and one was treated with systemic therapy. The remaining patients had distant metastasis, 1 of whom underwent resection of a solitary liver metastasis while 12 patients received systemic treatment.

Conclusions. Patients with completely resected stage IIIB/C melanoma have high risk of early recurrence before start of adjuvant therapy. Restaging should be considered for high-risk melanoma patients before start of adjuvant therapy.

Introduction

Treatment of stage III melanoma consists of complete resection with curative intent. However, the risk of recurrence afterwards is high, resulting in 5-year overall survival (OS) rates between 40% and 78%.[1-3] Therapeutic options and prospects for patients with metastatic melanoma have changed considerably in recent years, especially with the introduction of immune checkpoint inhibitors and BRAF- and MEK inhibitors.[4-10] These drugs have been proven to significantly improve OS in metastatic melanoma and have also shown promising results in the adjuvant setting. Phase III trials investigating adjuvant systemic therapy with ipilimumab (anti-CTLA-4 antibody) and combined dabrafenib/trametinib (BRAF/MEK-inhibitor) showed improved OS compared with placebo.[11, 12] Adjuvant nivolumab and pembrolizumab (both anti-PD-1 antibodies) led to improved 12-month recurrence-free survival (RFS) rates when compared with ipilimumab and placebo, respectively.[13, 14] Data on OS are still awaited. These results led to approval of ipilimumab, pembrolizumab, nivolumab, and combined dabrafenib/trametinib as adjuvant therapy by the Food and Drug Administration (FDA). The European Medicines Agency (EMA) approved use of nivolumab and combined dabrafenib/trametinib in the adjuvant setting and received a positive advice from the Committee for Medicinal Products for Human Use (CMHP) for adjuvant use of pembrolizumab.[15-23]

After diagnosis of nodal metastasis in high-risk stage III melanoma, imaging techniques [e.g. computed tomography (CT) or ^{18}F-fluorodeoxyglucose (^{18}F-FDG) positron emission tomography (PET)] are used to exclude distant metastasis. In stage IIIB/C melanoma, most recurrences appear within the first 2 years after surgical resection.[1] Despite this high risk, incorporation of imaging techniques in follow-up after resection differs widely between centers. No survival benefit of imaging during follow-up was demonstrated in a randomized trial, but this trial was carried out prior to the introduction of effective therapies for metastatic melanoma.[24, 25] In a clinical trial investigating adjuvant therapy, it is mandatory to exclude recurrent disease prior to inclusion, preventing metastatic melanoma patients from entering the adjuvant study.

We report herein imaging results for 120 stage IIIB and IIIC melanoma patients who underwent complete surgical resection within 12 weeks prior to inclusion in a randomized, placebo-controlled randomized trial investigating adjuvant dendritic cell therapy (NCT02993315). Imaging with contrast-enhanced venous-phase CT (ceCT) or ^{18}F-FDG PET/CT was performed to exclude recurrent disease within 6 weeks prior to inclusion.

Patients and methods

Patients

After signing informed consent, patients were screened for eligibility in a randomized placebo-controlled trial (NCT02993315) investigating adjuvant dendritic cell vaccination. The protocol has been approved by the national review committee (Central Committee on Research Involving Human Subjects) and is in concordance with the Declaration of Helsinki and Good Clinical Practice. Eligible patients were adults with stage IIIB or IIIC [American Joint Committee on Cancer (AJCC) 7th edition][2] cutaneous melanoma within 12 weeks after complete radical lymph node dissection (RLND) and after recovery from the surgery. The protocol was amended after publication of the MSLT-II trial results, which showed no survival benefit of completion lymph node dissection after removal of microscopic metastasis with sentinel node biopsy (SNB) when compared with nodal surveillance.[26] After amendment, patients with microscopic disease could be included after SNB and an additional completion lymph node dissection was no longer required. Macrometastasis was defined as a palpable node, a nonpalpable node of at least 15mm in short axis on CT, a PET-positive node, or one or more foci of melanoma of at least 1 cm in diameter in the pathology report. Patients with completely resected in-transit and/or satellite metastasis, an unknown primary tumor, and (planned) adjuvant radiotherapy could be included. In addition, absence of distant metastasis had to be documented by ceCT of the chest, abdomen and pelvis or whole-body ^{18}F-FDG PET scan combined with CT (^{18}F-FDG PET/CT) within 6 weeks before inclusion in our trial. In patients with head or neck melanoma, additional ceCT of the neck was obligatory. Imaging of the brain was performed in case of clinical suspicion of brain metastasis. Exclusion criteria included autoimmune disease (except for skin disease, hypothyroidism after autoimmune thyroiditis, and type 1 diabetes mellitus), a second malignancy in the last 5 years (except for adequately treated carcinoma in situ and basal or squamous cell carcinoma of the skin), concomitant use of oral or intravenous immunosuppressive drugs and uncontrolled infectious disease.

Methods

Within 6 weeks prior to the start of the study, imaging to exclude relapse was performed. Recurrence was considered symptomatic if suspected by symptoms and/or abnormalities during physical examination. Otherwise, recurrence was considered asymptomatic. Blood tests, including lactate dehydrogenase (LDH), were carried out within 4 weeks before inclusion. For baseline characteristics, a conglomerate of lymph nodes with at least four metastatic lymph nodes and presence of extracapsular extension was regarded as N3 disease. In case of a conglomerate, the diameter of lymph node involvement was counted as the diameter of the conglomerate.

Results

Patient characteristics

Between November 2016 and July 2018, 120 patients were screened for eligibility. Baseline characteristics are presented in **Table 1**. Median age was 54 (range 27-79) years, and 76 (63%) of patients were male. Sixty-nine (58%) and 51 (43%) patients were diagnosed with stage IIIB and IIIC melanoma, respectively. Twenty-one (18%) patients had completely resected in-transit metastasis, and 9 (8%) patients presented with nodal metastasis from an unknown primary tumor. Baseline characteristics of patients with and without recurrent disease during screening are presented in **Table 1**. No statistically significant differences between groups were present.

Detection of recurrent disease

Melanoma metastasis was detected in 22 (18%) of 120 patients (**Figure 1**), corresponding to a number needed to screen of 5.45, to detect one patient with recurrent disease. Thirteen (59%) patients were identified with distant metastasis, while in the remaining nine (41%) patients, metastasis was locoregionally located.

Five (23%) recurrences were found based on symptoms or physical examination (symptomatic recurrence); in 3 patients, in-transit metastasis was noticed by the patient (n = 1) or physician (n = 2), and another patient discovered a local recurrence at the site of the resected primary melanoma. Of these 4 patients with symptomatic locoregional relapse, 2 showed detectable distant metastatic disease on ceCT scan. The fifth patient developed back pain, which was suspicious for bone metastasis and confirmed by ceCT imaging. Seventeen (77%) relapses were asymptomatic and initially detected by imaging, corresponding to a number of asymptomatic patients needed to screen of 6.76. One of these patients presented with atypical, very small pulmonary nodules before RLND. Another patient showed atypical/nonspecific hypodense liver lesions of maximum 10 mm on preoperative ceCT scan, and these lesions were identified as liver metastases during screening ceCT after a 12-week interval. Serum LDH level was not a sensitive parameter for recurrent disease, since only 4 (18%) out of 22 relapsed patients had elevated LDH. All 4 patients had distant metastasis, and 2 of them were asymptomatic.

Table 1 Baseline characteristics

Characteristic	Total (n = 120)	No recurrent disease during screening (n = 98)	Recurrent disease during screening (n = 22)
Age			
Median (range) (years)	54 (27-79)	55 (27-79)	51 (27-73)
Sex, n (%)			
Male	76 (63)	59 (60)	17 (77)
Female	44 (37)	39 (40)	5 (23)
Stage at screening (AJCC 7th edition), n (%)			
IIIB	69 (58)	58 (59)	11 (50)
IIIC	51 (43)	40 (41)	11 (50)
Breslow, n (%)[a]			
<2mm	49 (44)	42 (47)	7 (32)
2-4mm	24 (22)	19 (21)	5 (23)
≥ 4mm	36 (32)	27 (30)	9 (41)
Other[b]	2 (2)	1 (1)	1 (5)
Ulceration, n (%)[a]			
Yes	38 (32)	31 (32)	7 (32)
No	73 (61)	58 (59)	15 (68)
Histological type, n (%)[a]			
Superficial spreading melanoma	73 (66)	61 (69)	12 (55)
Nodular melanoma	26 (23)	20 (22)	6 (27)
Other	7 (6)	5 (6)	2 (9)
Missing	5 (5)	3 (3)	2 (9)
Primary site, n (%)			
Head/neck	17 (14)	13 (13)	4 (18)
Trunk	46 (38)	37 (38)	9 (41)
Upper extremity	13 (11)	12 (12)	1 (5)
Lower extremity	34 (28)	26 (27)	8 (36)
Genital	1 (1)	1 (1)	0 (0)
Unknown primary	9 (8)	9 (9)	0 (0)

Table 1 Continued

Characteristic	Total (n = 120)	No recurrent disease during screening (n = 98)	Recurrent disease during screening (n = 22)
Type of lymph node involvement, n (%)			
Microscopic	21 (18)	19 (19)	2 (9)
Macroscopic	99 (83)	79 (81)	20 (91)
Maximum diameter of lymph node metastasis			
Median (range) (cm)	2.0 (0.01 – 7.5)	1.9 (0.01 – 7.5)	3.0 (0.25 – 7.0)
Number metastastic lymph nodes, n (%)			
0	1 (1)	1 (1)	0 (0)
1	46 (38)	40 (41)	6 (27)
2-3	37 (31)	29 (30)	8 (36)
≥ 4	36 (30)	28 (29)	8 (36)
Site of nodal metastasis, n (%)			
Neck	26 (22)	21 (21)	5 (23)
Axilla	51 (43)	46 (47)	5 (23)
Groin	42 (35)	30 (31)	12 (55)
Popliteal	1 (1)	1 (1)	0 (0)
Extracapsular extension, n (%)			
Yes	30 (25)	23 (23)	7 (32)
No	67 (56)	56 (57)	11 (50)
Missing	23 (19)	19 (19)	4 (18)
In-transit or (micro)satellite metastasis [c], n (%)			
Yes	21 (18)	17 (17)	4 (18)
No	99 (83)	81 (83)	18 (82)
BRAF, n (%)			
BRAF V600E/V600K	78 (65)	65 (66)	13 (59)
Wildtype	34 (28)	29 (30)	5 (23)
Other[d]	3 (3)	2 (2)	1 (5)
Missing	5 (4)	2 (2)	3 (14)

[a] Excluding 9 patients with unknown primary tumor, [b] Primary melanoma diagnosed as melanocytic tumor of uncertain malignant potential (MELTUMP) in 2 patients, confirmed by revision, [c] Including locoregional recurrences, [d] One inactivating mutation, one p.Leu485Trp mutation, one p.Thr599Dup mutation. *AJCC* American Joint Committee on Cancer, *BRAF* B-Raf proto-oncogene, serine/threonine kinase

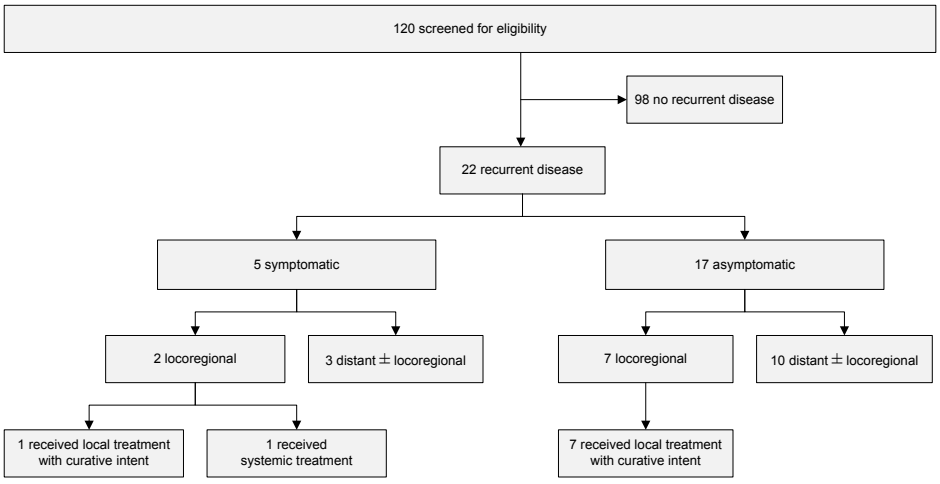

Figure 1 Detection of recurrent disease during screening for eligibility

Imaging techniques and intervals

Before referral to our trial, metastasis had been excluded with ^{18}F-FDG PET/CT (94%) or ceCT (6%) in 115 patients. Of the 5 patients in whom metastasis had not been excluded prior to screening, 4 had resected micrometastasis in the pathology report and one patient had macrometastatic disease. However, in all patients presenting recurrent disease during screening, distant metastasis had been excluded on imaging prior to start of screening for eligibility (**Figure 2**). For this group with early relapse, prior imaging was done using ^{18}F-FDG PET/CT in 20 patients (91%) and ceCT in the remaining 2 patients.

To screen for eligibility, 110 (96%) patients had standard ceCT. In the remaining 5 (4%) patients, imaging was performed by ^{18}F-FDG PET/CT. Relapse during screening was detected by ceCT in all cases. In 5 patients, imaging was not repeated during screening, since the start of experimental adjuvant therapy was within 6 weeks after prior imaging excluding distant metastasis.

The median interval between imaging during screening and previous imaging was 10.2 (range 5.7-20.9) weeks in recurrent patients. The median interval between complete resection and detection of recurrent disease was 7.4 (range 4.3-10.7) weeks. In patients without recurrent disease, these intervals were not significantly different, with a median interval between scans of 11.1 (range 5.6-27.7) weeks and an interval between resection and imaging of 7.3 (range 4.0-11.0) weeks. **Figure 3** shows examples of patients with asymptomatic recurrent disease.

Treatment of relapsed patients

Nine patients showed locoregional metastasis, of whom 8 were referred for surgical resection with curative intent. One patient had no evidence of disease after adjuvant radiotherapy, therefore planned surgery was cancelled. This patient was disease free during 13 months of follow-up, then relapsed. Of the 7 reoperated patients, 6 developed recurrent disease. In 2 of them, distant metastasis occurred within 1 month after resection of the recurrent local disease. In 4 patients, the interval from resection to recurrent disease was 6, 6, 8, and 9 months. The last reoperated patient is still recurrence free after 10 months of follow-up. In the remaining patient, locoregional recurrence consisted of irresectable in-transit metastasis, for which treatment with anti-PD-1 antibodies was initiated.

Of the 13 patients with distant metastasis, first-line treatment consisted of anti-PD-1 antibodies in 3 patients, 3 patients started with combined immune checkpoint inhibition, and in 6 patients treatment with targeted therapy was initiated. One patient underwent metastasectomy of a solitary liver metastasis.

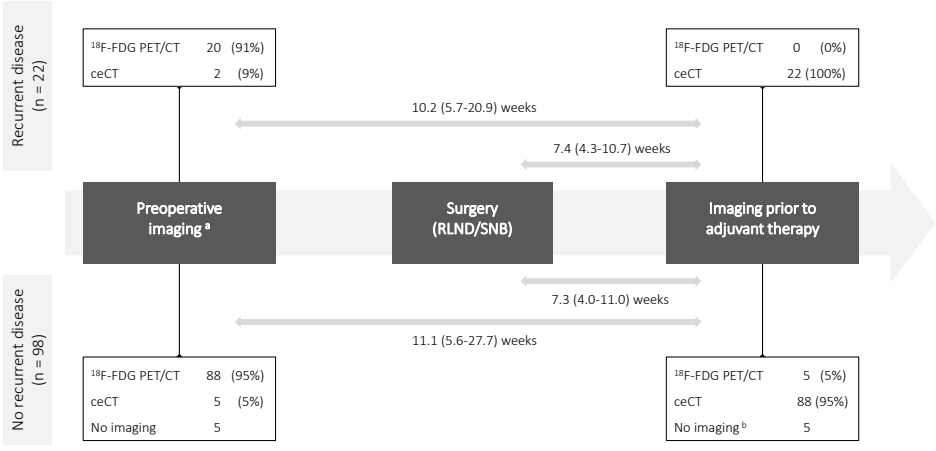

Figure 2 Time intervals and techniques of imaging used prior to intended start of adjuvant therapy
Time intervals are presented as median (range). [a] Imaging prior to referral for trial participation was performed postoperatively after sentinel node biopsy (micrometastatic disease) in nine patients and in three patients with macrometastatic disease. [b] Imaging was not repeated during screening for eligibility in five patients, since the inclusion in the adjuvant trial was within 6 weeks after prior imaging. *ceCT* contrast-enhanced venous phase CT, *18F-FDG PET/CT* 18F-fluorodeoxyglucose PET-scan combined with CT, *RLND* radical lymph node dissection, *SNB* sentinel node biopsy

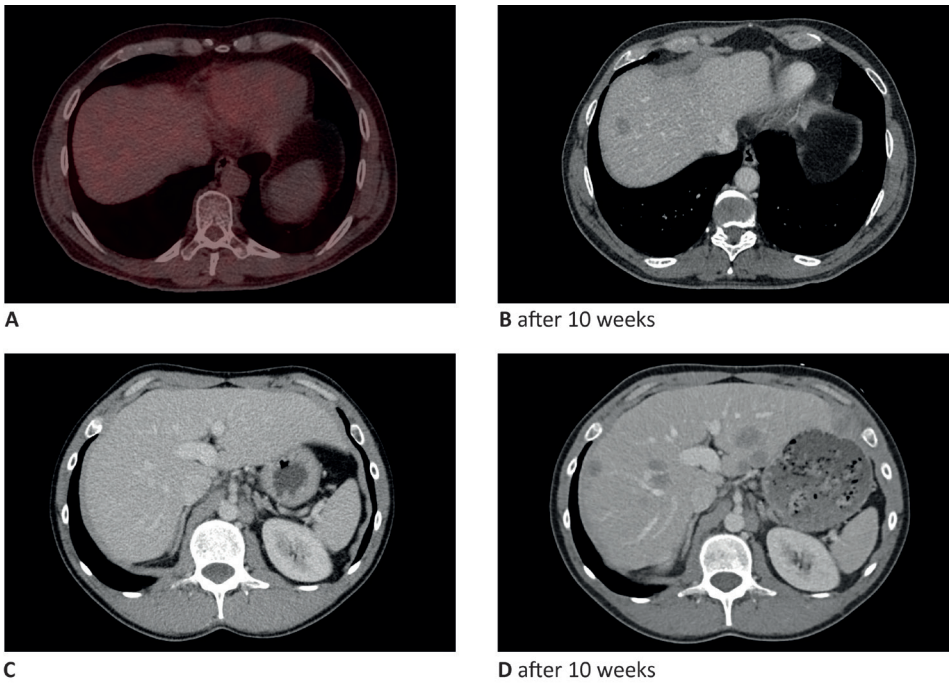

Figure 3 Asymptomatic recurrent melanoma during screening.
A patient with pT4aN1b/stage IIIB melanoma (AJCC 7th edition)[2]. Showed no metastatic disease on ^{18}F-FDG PET/CT prior to radical lymph node dissection (RLND) (**A**), but venous-phase contrast-enhanced CT (ceCT) 10 weeks after RLND and 12 weeks after prior ^{18}F-FDG PET/CT, showed liver metastasis (**B**). A patient with pT2aN2b IIIB melanoma (AJCC 7th edition). Showed no metastatic disease on ceCT (shown) and ^{18}F-FDG PET/CT (not shown) prior to RLND (**C**), but ceCT 5 weeks after RLND and 10 weeks after prior ceCT revealed multiple liver metastases (**D**).

Discussion

In 120 patients screened for an adjuvant trial, almost one out of 5 patients with completely resected stage IIIB or IIIC melanoma showed evidence of recurrent disease prior to start of adjuvant therapy, despite adequate prior imaging. These relapses were present within 2 months after surgery and within 3 months after previous staging. The majority of patients with recurrent disease were asymptomatic, and all were identified by ceCT scan.

Discovery of recurrent disease before start of adjuvant therapy improves information about prognosis. A proper baseline scan prevents incorrectly discarding of therapy if a metastasis is visualized at the first follow-up scan but was already present and detectable before start of therapy. In addition, evidence of relapse can change therapeutic management. About one-third of patients with recurrent disease were referred for additional resection with curative intent due to locoregional relapse. Furthermore, patients with a rapid relapse with relatively high metastastic load started treatment with BRAF/MEK inhibitors or combined anti-CTLA-4/anti-PD-1 antibodies. Therefore, reimaging before start of adjuvant therapy leads to a change in therapeutic management in a substantial group of patients and should be considered in all patients despite prior imaging.

A limitation of this study is that we only evaluated patients screened for eligibility in our clinical trial, hence a selection bias might have occurred. Patients with more unfavorable prognosis and higher risk of recurrence are more likely to be referred for trial participation than patients who would be referred for approved adjuvant treatment. On the other hand, some rapid relapses are missed in our report due to development of symptomatic metastasis or due to recurrent disease diagnosed at radiotherapy planning CT scans before screening for eligibility. The interval between scans was similar between the groups with and without relapse, therefore a lead-time bias does not seem to play a role.

To the best of the authors' knowledge, this is the first report about detection of early recurrent disease in resected stage III melanoma before start of adjuvant therapy. Studies have been conducted to analyze the discovery of metastasis by imaging in stage III melanoma patients during follow-up after resection.[27-33] However, these studies performed imaging during follow-up with a longer interval after surgery and did not report recurrences in relation to start of adjuvant therapy. Mostly, the first scan was conducted 6-12 months after surgery, thus information about rapid asymptomatic relapses within 12 weeks is lacking.

In line with our protocol, phase III trials investigating adjuvant treatment with anti-CTLA-4 or anti-PD-1 antibodies or BRAF/MEK inhibitors excluded metastasis with CT postoperatively and within 4–6 weeks prior to randomization.[11-14] The trial investigating adjuvant ipilimumab versus nivolumab reported that 24% of screened resected stage IIIB/C/IV patients no longer met criteria and were not randomized.[13] Exact numbers of screening failures due to recurrent disease were not mentioned but probably represent an important portion thereof. In addition, the contribution of relapse in stage IV melanoma patients, at higher risk for relapse than stage

IIIB/C patients, is not reported. It would be interesting to analyze the numbers of recurrent disease during screening in the larger study cohorts of adjuvant phase III trials.

Taken together, about one-fifth of completely resected stage IIIB/C melanoma patients had recurrent disease before start of adjuvant treatment. Because of the impact on prognosis and therapeutic consequences, restaging all high-risk patients before start of adjuvant therapy seems appropriate.

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6

Summarizing discussion and future perspectives

Adapted from Frontiers in Immunology, 2018.

Summarizing discussion and future perspectives

Melanoma and immunotherapy

Melanoma is a form of skin cancer which arises from melanocytes and potentially behaves aggressively due to its ability to metastasize. In early stages, the primary treatment consists of surgical resection with curative intent. For metastatic melanoma, dacarbazine, a chemotherapeutic agent, was the standard of care since 1972 (**Figure 1**) although it has never been tested in a placebo-controlled trial. With minor response rates of only 13% and little long-term benefit, median overall survival (OS) for metastastic melanoma at that time was between 6 and 11 months.[1] Despite all efforts, a superior chemotherapeutic drug or combination chemotherapy was not found and melanoma is considered to be a rather chemotherapy resistant tumor.[1]

Melanoma is considered an immunogenic type of malignancy due to reports of spontaneously regressed melanoma[2] which is regarded as an immune-mediated phenomenon. This drew attention of researchers with interest in cancer immunotherapy into this type of malignancy.[3] Cancer immunotherapy consists of drugs interfering with the immune response to eradicate tumor cells.[4] The immune response starts with antigen-presenting cells, such as dendritic cells (DCs) continuously patrolling the body for non-self (e.g. tumor) cells. After encountering a tumor cell, DCs pick up tumor antigens and migrate towards the lymph nodes. There they present tumor antigens to naïve T cells, generating a T cell response recognizing and eradicating tumor cells.[4]

Trials with different immunotherapies have been conducted, in the early years mainly investigating cytokines and active vaccination strategies.[5] The cytokine interleukin (IL)-2 was approved for the treatment of metastastic melanoma in 1998 based on durable response rates, although it never proved survival benefit and the high toxicity limits widespread usage.[6, 7] Interferon (IFN)-α was approved as an adjuvant therapy for melanoma, but without OS benefit and at the cost of significant toxicity.[8] Recently, the clinical benefit of adjuvant peptide vaccination was investigated in a large phase III trial. Patients with completely resected lymph-node involved (stage III) melanoma received combined administration of recombinant MAGE-A3, a cancer-testis antigen (CTA), and an immunostimulant. Unfortunately, the trial showed no survival benefit compared to placebo and no induction of cellular responses.[9] All efforts were finally rewarded with the approval of the first immune checkpoint inhibitor (ICI) in 2011, which meant a major breakthrough in cancer immunotherapy. ICI are a type of immunotherapy interfering with immune checkpoint (regulators) of the immunological response.[10] The monoclonal antibody (mAb) blocking the inhibitory immune checkpoint CTLA-4, ipilimumab, was the first ICI with proven survival benefit in metastastic melanoma patients and was approved in 2010.[11] A major advantage is the durable response seen in approximately 20% of patients. [12] In 2014 nivolumab and pembrolizumab, mAbs blocking the PD-1/PD-L1 inhibitory pathway, showed an even higher response rate and progression-free survival with less toxicity than ipilimumab. Toxicity is still

significant as around 10-22% of patients will encounter treatment-related grade 3-4 adverse events.[13-15] Combined ipilimumab and nivolumab generates higher response rates, however in terms of survival it has a more additive than synergistic effect. Toxicity is a major downside of the combination as 55% of patients experienced treatment-related grade 3-4 adverse events.[13, 16, 17] In preclinical and clinical research, the application of mAbs interfering with other inhibitory immune checkpoints, such as TIM-3, LAG-3, TIGIT or VISTA and control-stimulatory checkpoints such as OX40, ICOS and 4-1BB is explored.[18] In 2015, the intralesional oncolytic virus therapy talimogene laherparepvec (T-VEC) was added to the immunotherapeutic options for unresectable cutaneous, subcutaneous and nodal melanoma metastases. T-VEC is a herpes simplex virus genetically modified to only replicate in and lyse tumor cells and the GM-CSF gene is transfected for the local production of GM-CSF by the tumor cells leading to attraction of DCs. It showed improved durable response rates compared to GM-CSF alone.[19]

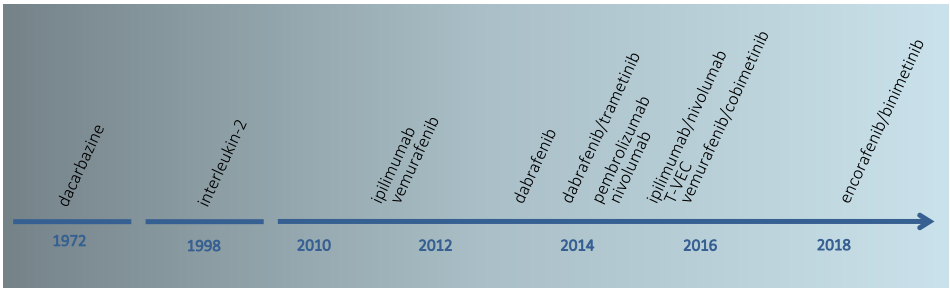

Figure 1 Evolving therapeutic landscape for metastatic and unresectable stage III melanoma

In addition to cytokines, peptide vaccination and ICI, immunotherapies using cell-based approaches are investigated: adoptive T cell therapy, chimeric antigen receptor (CAR) T cell therapy and DC vaccination. Briefly, in adoptive T cell therapy tumor-infiltrating lymphocytes harvested from tumor tissue are expanded *ex vivo* and reinfused intravenously after lymphodepleting chemotherapy. IL-2 is administered to prolong the persistence of infused cells.[20] This method has shown promising durable responses in metastatic melanoma patients in phase I/II trials and is currently investigated in a phase III trial (NCT0227887) in which adoptive T cell therapy is randomized against ipilimumab.[21-27] In CAR-T cell therapy autologous T cells are harvested via apheresis. Subsequently, T cell receptors (TCR) are genetically modified to consist of signaling chains of the TCR complex fused to an antigen-specific region of a single chain variable fragment from an antibody. After expansion these CAR-T cells are reinfused after lymphodepleting chemotherapy. This therapy has been studied mostly in hematological malignancies and showed high clinical response rates. For example, the CD19- specific CAR-T cell therapies tisagenlecleucel and axicabtagene ciloleucel are approved for the treatment of B-cell precursor acute lymphoblastic leukemia and large B-cell lymphoma, respectively,

by the Food and Drug Administration (FDA) and European Medicines Agency (EMA).[28] This established benefit contrasts with the so far rarely achieved clinical efficacy of CAR-T cells in solid tumors, thus further improvement is needed.[29] Phase I/II studies investigating CAR-T cell therapy in metastatic melanoma are ongoing.-

During DC vaccination, autologous (precursors of) DCs are used for the induction of an immune response. As described above, DCs play a pivotal role in the induction of immune responses and as such play an important role in the immune defense against cancer cells. DCs have been exploited for DC-based immunotherapy. Largely two decades ago the results of the first clinical trial testing the capacity of *ex vivo* matured and antigen loaded DCs in lymphoma were reported.[30] Until now, multiple clinical trials testing different forms of DC vaccination in numerous types of cancer have been enrolled. In 2010, the FDA approved the currently only registered therapeutic cancer vaccine, Sipuleucel-T, for the treatment of castration-resistant prostate cancer based on a 4-month benefit in OS without increased progression-free survival shown in a phase III trial.[31] This vaccine cannot strictly be regarded as a DC vaccine, due to the use of autologous mononuclear cells obtained by apheresis, with an end product containing a variety of cell types.[32]

For melanoma, the first results of a clinical trial with DC vaccination in metastatic melanoma were reported in 1998.[33] In 2006, a phase III trial in which patients were treated with DCs was stopped prematurely due to a lack of improved response rate compared to dacarbazine.[34] Thereafter, multiple improvements in the manufacturing process of DC vaccines were made. So far, clinical responses to DC vaccination are still sparse and a meta-analysis in 2014 showed a response rate of 8.5% in metastatic melanoma patients.[35] Contrary to the lack of proved survival benefit, induction of cellular immunological responses by DC vaccination is commonly reported.[35] An advantage of DC vaccination is that it, in contrast to the currently approved ICI, is associated with a mild toxicity profile, probably due to a tumor-specific induction of the immune response.[35] Attention of future research should focus on further optimizing DC-based immunotherapy to obtain more clinical responses. A hurdle for effective DC vaccination is an immune suppressive tumor microenvironment (TME) leading to cytotoxic T cell anergy after initial successful activation.[36] The position of DC vaccination therefore probably lies in the combination with therapies targeting factors suppressing the immune response. Another position of DC vaccination might be introduction in earlier disease stages, as tumor burden is very low hence tumor-induced immune suppression is almost absent, potentially improving clinical response to DC vaccination monotherapy.

In this thesis strategies to improve DC vaccination are described, including combination therapy, vaccination at an earlier stage of disease and improvement of the DCs used in vaccines. In addition, the favorable toxicity profile with specific attention for the health-related quality of life of patients is appointed. The last chapter focuses on the role of imaging prior to the start of adjuvant therapy which became important in the recently rapidly evolved therapeutic landscape of stage III melanoma.

Combination strategy

Tumors can evade the immunological anti-tumor response by diverse tumor-escape mechanisms, inducing immune suppression or avoiding immune recognition. For example, by the downregulation of MHC mediated tumor-antigen presentation, promotion of regulatory T cell (Treg) differentiation, accumulation of myeloid-derived suppressor cells (MDSCs) or upregulation of inhibitory checkpoint molecules such as PD-L1.[37] Reduction of tumor burden by surgery, radiation therapy, chemotherapy or targeted therapy can alleviate the tumor-induced immune suppression. However, possible synergy between therapies reducing tumor burden can involve more than the mere reduction of tumor load, as modalities other than immunotherapy also exhibit immunogenic effects on tumors (**Figure 2**).

Radiation therapy induces DNA strand breaks, apoptosis and necrosis, but also immunogenic modulation for example through the upregulation of MHC molecules and immune-activating cytokines recruiting DCs and by immunogenic cell death.[38] Through this immune priming effect of radiotherapy, combination therapy with DC vaccination[39] or ICI might have a synergistic effect[40]. Chemotherapeutic drugs are also known to have immunomodulatory properties. For example, cisplatin has shown immunomodulatory properties *in vitro* and *in vivo* as it suppresses inhibitory factors in the TME, such as Tregs and MDSCs and it induces immunogenic cell death.[41, 42] To study the synergistic potential of cisplatin and DC-based immunotherapy we conducted a randomized phase II clinical trial in which 54 stage III and IV melanoma patients were treated with combined DC vaccination and cisplatin or DC vaccination monotherapy. The combination therapy induced immunological response and was feasible and safe (**Chapter 2**). However, we could not find a benefit of the addition of cisplatin to DC vaccination as there was no difference in immunological or clinical response between both treatment groups. Two possible explanations for the lack of synergy are the timing of treatment and the administration of dexamethasone. The timing of cisplatin relative to the vaccination might have been suboptimal and might be improved by a longer interval between cisplatin and vaccination.[43] Dexamethasone, which was administered as an antiemetic drug, can diminish the proinflammatory immune response to DC vaccination. However, to date it is still unknown whether steroids interfere with response to immunotherapy.

Platinum-based chemotherapeutics are tested in clinical trials combined with the ICI anti-PD-1 and anti-PD-L1 mAbs, for example in non-small lung cancer.[44] Because of the rather chemoresistant nature of melanoma, the combination of chemotherapy and immunotherapy is less likely to be tested in melanoma. At time of writing, one ongoing phase III trial investigating combination of ICI with chemotherapy in melanoma was registered. In this trial (NCT02460068) ipilimumab monotherapy is compared to ipilimumab in combination with fotemustine, an alkylating agent with immunomodulatory properties.[45]

Targeted therapy with BRAF and MEK inhibitors leads to an improved progression-free and overall survival in patients with metastatic melanoma harboring an activating BRAFV600 mutation. Three combinations of BRAF and MEK inhibitors are currently approved: vemurafenib/

cobimetinib, dabrafenib/trametinib and encorafenib/binimetinib.[46-50] Besides a rapid reduction in tumor burden, BRAF/MEK inhibition can induce CD8+ T cell infiltration in tumor tissue.[51] This might potentiate the effect of other immune therapies, such as DC vaccination.[52] Combination of BRAF/MEK inhibitors and ICI is tested in a number of phase I/II trials and in one ongoing phase III trial (NCT02967692) investigating dabrafenib/trametinib with or without spartalizumab (anti-PD-1 mAb).

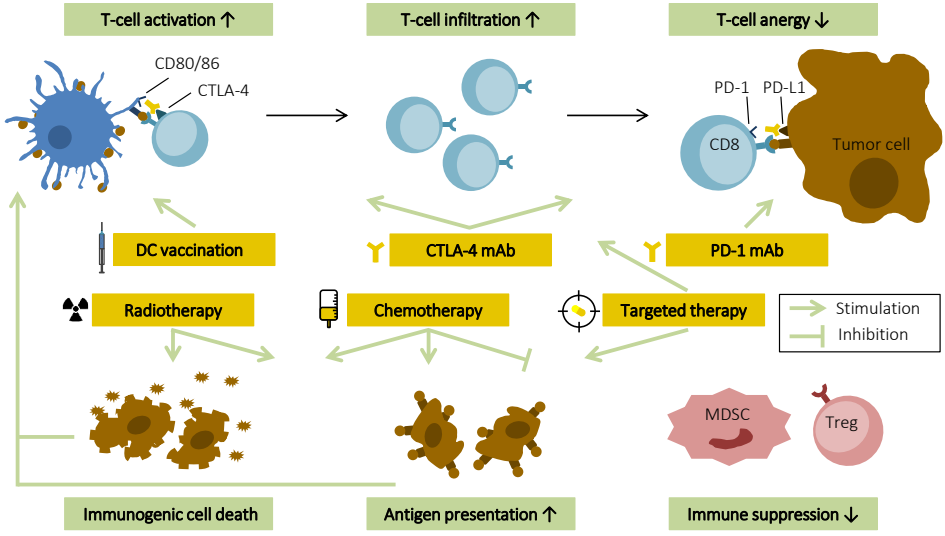

Figure 2 Combinational strategies to achieve synergy between several treatment modalities and dendritic cell vaccination. *mAb* monoclonal antibody, *MDSC* myeloid derived suppressor cells, *Treg* regulatory T cells

Besides combination of immunotherapy with radiation therapy, chemotherapy or targeted therapy, the combination of different immunotherapies has been studied as well. For example, the combination of different ICI. In metastatic melanoma, combined ipilimumab and nivolumab led to a higher response rate, at the cost of considerably increased toxicity and without a clear synergistic effect.[16, 17] A large number of other combinations of ICI are investigated in phase I/II trials[53] and one phase III trial (NCT03470922) is ongoing comparing the combination of nivolumab and relatlimab, an anti-LAG-3 mAb, to nivolumab monotherapy. Other immunomodulating agents such as an IDO-inhibitor, a colony-stimulating factor, an IL-2 agonist specifically inducing CD8+ T cell differentiation and a TLR9 agonist are also currently tested in phase III trials in combination with nivolumab and/or ipilimumab (NCT03329846, NCT02339571, NCT03635983 and NCT0344553). Furthermore, the oncolytic virus therapy T-VEC is investigated in a phase III trial (NCT02263508) in combination with pembrolizumab. Besides of combined nivolumab/ipilimumab, results of one phase III trial investigating

combined immunotherapy results are known at time of writing. This trial, in which the IDO-inhibitor epacadostat was added to pembrolizumab, was stopped since it showed no survival benefit compared to pembrolizumab monotherapy.[54] This was despite promising phase I/II data, therefore results of phase III trials investigating combination therapies need to be awaited.

As demonstrated by the combination of ipilimumab and nivolumab, it is important to take the risk of increased toxicity into account when combining different therapies. The favorable toxicity profile of DC vaccination makes this therapy an ideal combinatorial partner. In an earlier trial, DC vaccination was combined with daclizumab, an anti-CD25 mAb, to deplete Tregs. Despite depletion of peripheral Tregs, the combination failed to show superiority, probably due to the concomitant depletion of functional $CD25^+$ effector cells.[55] The ICI anti-CTLA-4 mAb and anti-PD-1 mAbs were approved for metastatic melanoma patients after the conduction of the trial with cisplatin described in **Chapter 2**. These might be better candidates than cisplatin to combine with DC vaccination in melanoma patients. For response to anti-PD-1 mAbs, tumor-specific T cells have to be present in the TME, the generation of which may be aided by DC vaccination.[56] Conversely, T cells induced by DC vaccination are often hindered by the immune suppressive milieu of tumors. ICI might aid the effector functions of these T cells by blocking the inhibition of activated T cells through PD-1/PD-L1 signaling or by enhancing T cell activation through the modulation of the CTLA-4/CD28-axis. Combining ICI with DC vaccination might therefore result in a synergistic immunological anti-tumor effect. In 2009, Ribas et al. reported safety of combining tremelimumab (anti-CTLA-4 mAb) and DC vaccination in 16 melanoma patients.[57] The trial was designed as an escalation study for tremelimumab in which 25% of patients achieved an objective clinical response. This is higher than the 11% found in the phase III trial with tremelimumab.[58] In addition, Wilgenhof et al. showed a promising overall response rate of 38% in 39 metastatic melanoma patients treated with the combination of ipilimumab and DC vaccination.[59] In 36% of patients grade 3 or 4 adverse events were seen, which is comparable with rates seen in large clinical trials with monotherapy ipilimumab.[60, 61] This suggests possible improved responses with little added toxicity from the addition of DC vaccines to ICI. Instead of anti-CTLA-4 mAbs, anti-PD-1 mAbs might be more suitable combinatorial partners for DC vaccination, considering their lower toxicity and better response rates. As of now, no data on combined anti-PD-1 mAb and DC vaccination have been published. However, several clinical trials investigating combinations of DC vaccination with clinically approved ICI including anti-PD1 mAbs are currently being performed in melanoma and multiple other tumor types.[62]

In conclusion, combination strategies are an important focus of current and future research. Incorporating DC vaccination in combinational strategies for the treatment of cancer is a promising field of research. Considering the favorable results on the combination of DC vaccination and anti-CTLA-4 mAb, the results on the currently ongoing combinational clinical trials, mainly with anti-PD-1 mAbs, are eagerly awaited.

DC vaccination as adjuvant therapy

Besides combination therapy, another strategy to overcome tumor-induced immune suppression, is the introduction of DC vaccination at an earlier stage of disease.[63] More antigen-specific T-cells were found after DC vaccination in stage III compared to stage IV melanoma patients.[64] A retrospective analysis of stage III melanoma patients included in different phase I/II DC vaccination trials shows that patients treated with adjuvant monocyte-derived DCs (moDCs) had a significantly improved OS compared to matched controls.[65] Therefore, we conducted a prospective trial investigating DC vaccination in stage III melanoma patients (**Chapter 3**). In this trial, the DC vaccination was potentially improved in different ways. Until recently, in most studies moDCs were used, DCs that are derived *ex vivo* from autologous monocytes harvested by apheresis. The differentiation process takes around 5-9 days in the presence of large amounts of cytokines: IL-4 and GM-CSF. Those cytokines can attenuate the efficacy of the generated DCs.[66, 67] Nowadays, it is possible to directly select the scarce naturally occurring DCs from the blood, obliviating the intensive culture period.[68] There are two major subsets of naturally occurring DCs (nDCs) that can be isolated from the blood: myeloid or conventional DCs (mDCs) and plasmacytoid DCs (pDCs). MDCs can be further subdivided into CD1c (BDCA-1) expressing mDCs and a minor subset expressing CD141 (BDCA-3).[69] In metastatic melanoma, vaccination with $CD1c^+$ mDCs or pDCs led to immunological response and long-term survival in some patients.[70, 71] After the conduction of these trials, multiple potential improvements to the production process have been made. Both subsets can be stimulated by protamine-mRNA complexes into functional mature DC.[72] Furthermore, prior to the selection of $CD1c^+$ mDCs, $CD14^+$ cells were depleted to exclude $CD14^+$ $CD1c^+$ cells that attenuate vaccine efficacy.[73] In addition, the pool of antigens used to load the DCs was expanded with CTAs frequently present in melanoma.[74] Lastly, we hypothesized that the concurrent administration of $CD1c^+$ mDCs and pDCs in one vaccine might be superior to one of the subsets alone. MDCs and pDCs possess a distinct phenotype, have a different capacity in pathogen detection and they produce different cytokines.[75, 76] Therefore, administration of both subsets might further enhance immunological and clinical response. In a murine model, the combination was indeed superior in reducing tumor size and increasing survival compared to either one of the subsets alone.[77] We conducted a randomized phase II clinical trial comparing vaccination with combined $CD1c^+$ mDCs and pDCs with $CD1c^+$ mDCs or pDCs alone in 15 stage III melanoma patients and results thereof are described in **Chapter 3**. After administration of $CD1c^+$ mDCs and pDCs, immunological responses were seen in 4 out of 5 (80%) of patients. The production was feasible and administration safe as we found no unexpected and only mild toxicity. Although groups were too small to draw major conclusions, at least the combination does not seem to be unfavorable compared to the administration of the single DC subsets.

Future improvements of DC vaccination might be the incorporation of $CD141^+$ (BDCA-3) mDCs. This minor subset has an important role in cross presentation of necrotic cell-derived antigens and activation of $CD8^+$ T cells.[78-80] When the process of selecting these cells is optimized and ready for clinical application, experimental therapy with this subset

in conjunction with $CD1c^+$ mDCs and pDCs is of interest.[81] Another possible improvement might be the adjustment of the antigens used for antigen loading of the DCs. NDCs in the trial described in **Chapter 3** were not only loaded with the tumor-associated antigens gp100 and tyrosinase, but also with the cancer-testis antigens MAGE-C2, MAGE-A3 and NY-ESO-1. Because the cancer-testis antigens used induced immunological responses more frequently than the tumor-associated antigens, the addition thereof might be important. Besides broadening of the number of antigens used, the DCs were loaded with peptide pools with overlapping peptides covering the complete sequence of the antigen. We found more immunological responses against these peptide pools than the corresponding peptides, showing the value of using overlapping peptide pools to load the DCs.

In addition, the use of neoantigens for antigen loading is of interest. Neoantigens arise from tumor mutations leading to novel tumor-specific proteins and T cells recognizing these proteins evade central T cell tolerance.[82] Immunotherapy with these highly personalized antigens likely possesses high specificity and an improved safety profile due to the avoidance of off-target toxicity. Disadvantages are the necessity of resected tumor tissue, the selection of the most immunogenic neoantigens and the difficult and time-consuming manufacturing procedure to obtain the neoantigen-specific vaccines. An exploratory study showed feasibility and immunogenicity of DCs loaded with neoantigens in 3 melanoma patients.[83] Another study investigated the use of neoantigens as adjuvant peptide vaccination in 6 stage IIIB/C melanoma patients which also showed immunogenicity and 4 of the patients had a recurrence-free survival lasting for at least 2 years.[84] Another ongoing development is the use of *in vivo* targeting instead of *ex vivo* loading of DCs, thereby circumventing the expensive and time-consuming generation period of personalized DC vaccines in a dedicated infrastructure.[85] Other advantages are the targeting of different DC subsets simultaneously and a more off-the-shelf product. The *in vivo* priming of DCs can be achieved in different ways, for example with nanoparticles containing antigens and adjuvants promoting DC maturation.[86] Current data mainly consists of preclinical studies reporting feasibility and safety of *in vivo* DC targeting melanoma cells in mice.[87-89] In addition, clinical data is available, as for example Dhodapkar et al. showed that *in vivo* targeting of DC was feasible, safe and showed biological activity.[90] In conclusion, these are promising developments of which further research needs to be awaited.

Position of DC vaccination in the evolving landscape of adjuvant therapy

At present, the adjuvant therapeutic options for stage III melanoma have expanded as multiple phase III trials investigating adjuvant therapy showed a positive impact on clinical outcome. Ipilimumab showed an improved 5-year OS rate compared to placebo and is approved by the FDA for clinical application.[60, 91] Targeted therapy with combined dabrafenib/trametinib is approved by both the FDA and the EMA based on favorable 3-year OS.[92] As for targeted therapy, nivolumab and pembrolizumab have been approved by both registration authorities after nivolumab and pembrolizumab showed 1-year recurrence-free survival benefit compared

to ipilimumab and placebo, respectively.[93, 94] The adjuvant application of ipilimumab and nivolumab together is also investigated in a phase III trial (NCT03068455), however this will likely be at the cost of higher toxicity, as is the case in the metastastic setting.[13] The clinical benefit of combined mDC and pDC vaccination as adjuvant therapy in stage III melanoma is currently investigated in our placebo-controlled randomized phase III trial (NCT02993315). This trial also collects data on health-related quality of life (HRQoL) during and after DC vaccination. Median age of patients diagnosed with melanoma is 59 years[95] and a considerable part of melanoma patients is thus of working-age. In addition, half of stage III melanoma patients will not encounter recurrent disease, but if treated, are exposed to the potential harmful side-effects of adjuvant therapy without any survival benefit. Therefore, taking toxicity and HRQoL into account is necessary.

Administration of combined dabrafenib/trametinib and ipilimumab is associated with high toxicity as grade 3 or 4 adverse events were experienced by 41% and 54% of patients, respectively.[91, 92] For ipilimumab, treatment-related adverse events occurred in 42-46% of patients.[91, 93] Although nivolumab and pembrolizumab show less toxicity, grade 3 or 4 adverse events are still reported in 25% of patients and these are treatment-related in 14-15% of patients.[93, 94] Patients treated with adjuvant ipilimumab and pembrolizumab reported a non-clinically relevant decline in HRQoL during treatment, whilst recovery from the radical lymph node dissection is expected.[96, 97] HRQoL of patients treated with adjuvant nivolumab or dabrafenib/trametinib remained unchanged.[93, 98] An advantage of DC vaccination is the mild toxicity profile which is translated into a significantly and clinically relevant improved HRQoL during adjuvant DC vaccination, as shown in **Chapter 4**. These data suggest that recovery from the radical lymph node dissection, performed within 12 weeks prior to the start of therapy, is not hampered by DC vaccination. This is supported by earlier reports stating DC vaccination did not impact the HRQoL in other tumor types.[99, 100] This finding needs to be confirmed in a larger cohort of patients and compared with a control group, as aforementioned this data is currently collected in our ongoing phase III trial. The toxicity profile of DC vaccination and lack of negative impact on HRQoL make DC vaccination an ideal candidate for adjuvant treatment. However, prior to positioning DC vaccination as an adjuvant treatment option for stage III melanoma, the results of the phase III trial investigating the clinical benefit of DC vaccination have to be positive. Despite early termination of the enrollment phase, the results of approximately 150 included patients is expected at the beginning of 2021. In addition, the results on OS benefit of adjuvant nivolumab and adjuvant pembrolizumab are still expected.

In this changing therapeutic landscape, it is also important to select the right patient for the right treatment. Only a portion of patients will suffer recurrent disease and benefit from adjuvant therapy. To date, only the tumor characteristics as defined in the American Joint Committee of Cancer (AJCC) classification are used for this purpose, however patient heterogeneity within substages IIIA/B/C is significant.[101] Other prognostic biomarkers are needed to further define the risk for the individual patient, thereby omitting unnecessary

toxicity and costs of therapy. In the future, circulating tumor DNA might be used for patient selection since it has already displayed some prognostic value in stage III melanoma.[102-104] Predictive biomarkers facilitate choice of therapy as only a minority of patients will have benefit of the currently available therapies. Besides the presence of a BRAF V600 mutation for response to BRAF/MEK inhibition, other predictive factors are not defined at this moment. PD-L1 expression and tumor mutational burden are not predictive for response to anti-PD1 mAbs in melanoma, as responses are seen in patients with low PD-L1 expression or low mutational burden as well.[105] Essential future research should be directed to further explore factors such as the different immune cells present in the tumor immune landscape, the upregulation of different checkpoint molecules and the tumor mutational burden. The advantage of the latter is the possibility for assessment in liquid biopsies, while the first require tumor tissue. At present, no prognostic biomarker for risk of recurrent melanoma within the AJCC substages and no biomarker predictive for response to ICI are defined. Most likely, such a future prognostic and predictive biomarker will consist of a combination of different factors.

Adjuvant therapy in stage III melanoma - implications for imaging

In the current evolving therapeutic era of adjuvant therapy for stage III melanoma, an important aspect is to reassure the absence of detectable distant metastases prior to the start of treatment as this might have therapeutic consequences. It is known that stage IIIB and IIIC melanoma recurs mostly in the first years after the excision of lymph node metastases.[106] Trials investigating recurrent disease on imaging during follow-up after surgical resection have been published.[107-114] However, there were no published data about the proportion of patients showing a relapse as early as upon the anticipated start of adjuvant therapy (within 12 weeks after surgery). Consequently, it was not known whether renewed staging after surgical resection is appropriate. In **Chapter 5** the results of imaging during the screening of patients for eligibility in our phase III trial investigating adjuvant DC therapy are presented. Of the 120 stage IIIB/C melanoma patients (AJCC 7th edition[101]) we screened, 18% displayed detectable recurrent disease at a CT scan during screening. In all early relapsed patients, metastases had been excluded prior to the lymph node surgery, mainly by FDG-PET. Of the relapses, 77% were asymptomatic and thus would have stayed undetected if no additional scan was made. Since, in our data, the early recurrences occurred both in subgroups with relatively high-risk and low-risk features, we recommend to restage all IIIB/C melanoma patients prior to the start of adjuvant therapy because this can have important implications for the choice of therapy. In addition, a proper baseline scan prevents the wrongful discarding of adjuvant therapy when metastases are visualized during the first follow-up scan, but were already present and detectable prior to the start of therapy. It is of interest to confirm this finding in a larger cohort. In addition to our data, studies investigating the frequency and duration of imaging during adjuvant therapy are needed to incorporate imaging in a cost-effectiveness manner.

General conclusion

DC vaccination induced immunological response in multiple trials, however, until now clinical benefit is found only in a minority of patients. This might be the result of diminished T cell infiltration in the tumor, immunosuppressive factors such as Tregs and MDSCs, T cell exhaustion or upregulation of inhibitory checkpoint molecules. A role for DC vaccination as monotherapy in metastastic melanoma is therefore unlikely and DC vaccination in combination with agents tackling immune suppressive factors needs to be further explored. The immunomodulatory chemotherapeutic agent cisplatin did not suggest to be of additional benefit to DC vaccination. Besides, nowadays multiple effective agents are approved for use in melanoma. Further research in melanoma should focus on DC vaccination in combination with other agents, and combination with anti-PD-1 mAbs is a logical next step. In addition to combination strategies, the incorporation of DC vaccination at an earlier stage of disease might also circumvent the presence of immune suppressive factors. The mild toxicity profile and lack of negative impact on HRQoL make DC vaccination an ideal adjuvant treatment option. Adjuvant treatment of stage III melanoma took an enormous flight and to date several systemic treatment options with survival benefit are available. Results of the phase III trial investigating the clinical benefit of adjuvant DC vaccination are awaited. In addition, the OS data of adjuvant anti-PD-1 mAbs are awaited. Future research should focus on personalized treatment strategies in this evolving field of therapeutic options. First, to select patients at highest risk for recurrent disease and therefore in need of adjuvant therapy. Second, to select a therapy the patient is likely to respond to. Lastly, for both combination and adjuvant therapy, the optimization of the DC vaccine remains of utmost importance with the exploration of antigen loading with neoantigens and *in vivo* targeting as promising fields of research.

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Het melanoom is een vorm van huidkanker die ontstaat uit de pigmentvormende cellen (melanocyten) in de huid. Doordat een melanoom kan uitzaaien (metastaseren) is het een potentieel dodelijke aandoening en daardoor een agressieve vorm van huidkanker. Tot ongeveer tien jaar geleden overleed bijna iedereen die de diagnose gemetastaseerd melanoom kreeg. Het is dan ook heel bijzonder dat tegenwoordig bij een deel van de patiënten de ziekte jarenlang -en mogelijk permanent- verdwijnt.

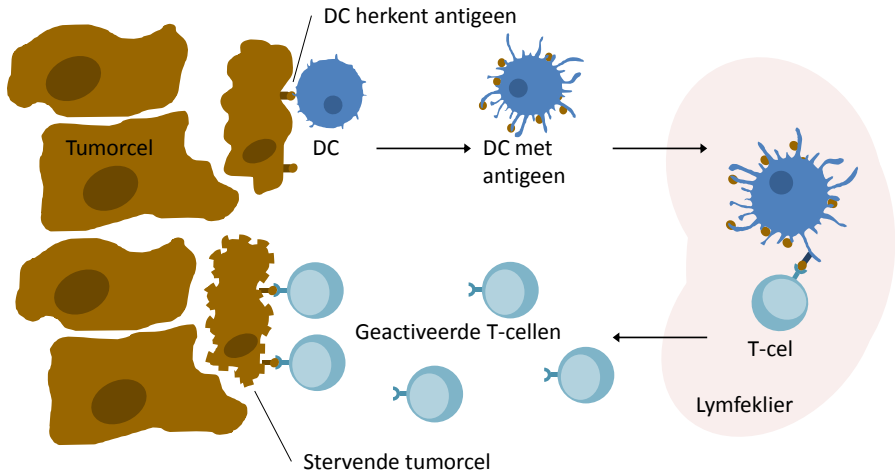
Dat de prognose zo enorm verbeterd is, is te danken aan twee belangrijke groepen medicijnen: doelgerichte therapie en immuuntherapie. Daarnaast onderzoekt men of de huidige behandelmogelijkheden verder uitgebreid kunnen worden. Bijvoorbeeld met dendritische cel (DC) vaccinatie.

Interactie tussen tumoren en het immuunsysteem

Eind 19de eeuw was William Coley al geïnteresseerd in het gebruik van het eigen afweersysteem (immuunsysteem) voor de behandeling van kanker. Hij bestudeerde enkele patiënten waarbij tumoren verdwenen nadat ze een infectie hadden doormaakten en schreef dit toe aan de reactie van het immuunsysteem. De onderzoeken waarin hij kankerpatiënten behandelde door het introduceren van een infectie om zo het immuunsysteem te stimuleren, waren toentertijd controversieel. Sinds deze eerste stappen in de ontwikkeling van immuuntherapie, heeft hier veel preklinisch en klinisch onderzoek naar plaatsgevonden. Inmiddels weten we dan ook veel beter hoe het immuunsysteem reageert op tumorcellen.

Tumorcellen zijn, net als bacteriën en cellen die geïnfecteerd zijn door een virus, voor het immuunsysteem lichaamsvreemde cellen die moeten worden vernietigd. Doordat elke cel kenmerkende eiwitten (antigenen) op de buitenkant draagt, kan het immuunsysteem cellen herkennen en ze onderscheiden in lichaamseigen en lichaamsvreemd. Bij tumorcellen zijn veranderingen (mutaties) opgetreden in het DNA, waardoor ook de antigenen aan de buitenkant van de cel veranderd zijn en deze cellen gezien worden als lichaamsvreemd. De immuunreactie begint met DC's; dat zijn antigeen-presenterende cellen (**Figuur 1**). DC's zijn continu in heel het lichaam op zoek naar lichaamsvreemde cellen, in dit geval tumorcellen. Wanneer ze tumorcellen tegenkomen, nemen ze tumorantigenen van de buitenkant van de tumorcel in zich op, worden ze volwassen (matuur) en vertrekken naar een lymfeklier. In de lymfeklier kunnen ze de rest van het immuunsysteem activeren. T-cellen spelen hierin een belangrijke rol. De DC's laten in de lymfeklier aan de T-cellen zien welke lichaamsvreemde tumorantigenen ze aantroffen. De T-cellen die deze tumorantigenen herkennen worden actief en gaan zich vermenigvuldigen. De vele tumorspecifieke T-cellen die daardoor ontstaan verlaten de lymfeklier en gaan via de bloedbaan op zoek naar cellen met deze tumorantigenen. Wanneer de T-cellen de tumorcellen tegenkomen, zullen ze de tumor binnendringen en de tumorcellen vernietigen.

Dat tumoren ontstaan betekent dat de hiervoor beschreven tumor-immuniteitscyclus niet altijd optimaal functioneert. Het kan op verschillende plekken in de cyclus fout gaan. Bijvoorbeeld doordat DC's de tumor niet herkennen, de DC's niet goed matureren, ze de T-cellen niet goed activeren, of de T-cellen de tumor niet binnendringen, herkennen of vernietigen. De aanwezigheid van cellen die het immuunsysteem onderdrukken (immuunsuppressie) of cellen die meer immuunsuppressieve moleculen tot expressie brengen kan hiervan een oorzaak zijn. Immuuntherapie is een verzamelnaam voor anti-tumor behandelingen die de tumor-immuniteitscyclus versterken in de reactie tegen tumorcellen.



Figuur 1 Anti-tumor immuunrespons
Dendritische cellen herkennen tumorcellen aan de antigenen op de buitenkant van de cel. Ze matureren en nemen de tumorantigenen mee naar een lymfeklier. In de lymfeklier worden de antigenen gepresenteerd aan T-cellen, die hierdoor actief worden en zich gaan vermenigvuldigen. Vervolgens vertrekken de T-cellen uit de lymfeklier en gaan via de bloedbaan op zoek naar cellen met de betreffende tumorantigenen aan de buitenkant. De T-cellen verlaten de bloedbaan en infiltreren de tumor. Daarna zetten ze de aanval in en vernietigen ze de tumorcellen. Wanneer tumorcellen doodgaan komen meer tumorantigenen vrij die de dendritische cellen kunnen oppakken en waardoor de reactie verder gaat. *DC* dendritische cel.

Immuuntherapie bij het melanoom

Bij het melanoom is veel onderzoek naar immuuntherapie gedaan omdat het melanoom goed door het immuunsysteem kan worden herkend. Men kwam dit op het spoor doordat in de literatuur is beschreven dat bij enkele patiënten het melanoom spontaan, dus zonder een vorm van (effectieve) behandeling, is verdwenen. Ook bij patiënten waarbij de ziekte al was gemetastaseerd, is dit gebeurd. Dit komt waarschijnlijk doordat het eigen immuunsysteem de melanoomcellen heeft vernietigd.

Immuuntherapie is de afgelopen decennia op verschillende manieren onderzocht, bijvoorbeeld door een algehele stimulatie van de immuunreactie met de eiwitten interleukine-2 of interferon-alfa. Dit zijn beide middelen met forse bijwerkingen en slechts een minimaal effect op de tumorgroei. In 2011 was er een doorbraak in de immuuntherapie door de registratie van ipilimumab, de eerste immune checkpoint inhibitor. Immune checkpoint inhibitoren zijn medicijnen die een bepaalde rem van het immuunsysteem halen. Het immuunsysteem heeft verschillende remmende moleculen om overactiviteit te voorkomen. Tumoren kunnen deze remmen misbruiken om te ontsnappen aan het immuunsysteem. Ipilimumab blokkeert het remmende molecuul CTLA-4, waardoor de T-cellen beter geactiveerd kunnen worden. In 2014 is een tweede soort immune checkpoint inhibitoren geregistreerd, deze blokkeren het remmende molecuul PD-1. Door het opheffen van deze rem kunnen de T-cellen de tumorcellen beter aanvallen. Zowel het middel pembrolizumab als nivolumab zijn PD-1-remmers. PD-1-remmers leiden vaker tot afname van de tumorgroei en hebben minder bijwerkingen dan ipilimumab. Een deel van de patiënten heeft een jarenlange aanhoudende reactie na behandeling met een immune checkpoint inhibitor. Hoewel het onderzoek ernaar nog lopende is, is het goed mogelijk dat deze patiënten door de immuuntherapie genazen. Dat de ontdekking van immune checkpoint inhibitoren een doorbraak betekende voor de behandeling van kanker, is ook af te leiden uit het feit dat James P. Allison en Tasuku Honjo in 2018 de Nobelprijs voor Fysiologie of Geneeskunde ontvingen voor hun ontdekking van de remmende moleculen CTLA-4 en PD-1.

Naast immuuntherapie is de registratie van doelgerichte therapie een andere belangrijke doorbraak in de behandeling van het gemetastaseerd melanoom. Dat werkt voor melanomen met een mutatie in het BRAF-gen, verantwoordelijk voor het aanmaken van het bij de celdeling betrokken BRAF-eiwit. Bij patiënten met een mutatie in het BRAF-gen werkt dit eiwit te hard, waardoor de cellen overmatig delen. Remming van het BRAF-eiwit met doelgerichte therapie is effectief. Helaas leidt de behandeling uiteindelijk tot ongevoeligheid waardoor de meeste patiënten maximaal negen maanden reageren. Het MEK-eiwit is net het als BRAF-eiwit betrokken bij de celdeling, alleen net verderop in de signaaloverdracht. Wanneer naast het BRAF-eiwit ook het MEK-eiwit wordt geremd, kan de celdeling langere tijd onderdrukt blijven. Bij een deel van de patiënten kan de groei van het melanoom door de combinatie van een BRAF en MEK-remmer een aantal jaren geremd blijven. Op dit moment zijn de onderzoeksresultaten nog niet zo ver dat al bekend is hoe lang dit effect aan kan houden.

Adjuvante behandeling stadium III melanoom

Van 1972 tot 2011 bestond de behandeling van een gemetastaseerd melanoom uit chemotherapie met het middel dacarbazine. De kans op aanslaan van de behandeling was echter slechts 5 tot 15 procent en met name de kans op langdurig profijt van de behandeling was erg laag. Zowel de CTLA-4 en PD-1-remmers als de BRAF en MEK-remmers veranderden de prognose voor patiënten met een gemetastaseerd melanoom enorm en geven de

patiënten die reageren op de behandeling goede vooruitzichten. Desondanks heeft het melanoom, wanneer gemetastaseerd, ook tegenwoordig een ongunstige prognose. Dat komt doordat niet iedereen (langdurig) reageert en uiteindelijk helaas de meerderheid van de patiënten zal overlijden. Daarom blijft het van belang metastasen in de organen te voorkomen door het melanoom al vroeg door de dermatoloog en/of chirurg te laten verwijderen. Ook wanneer het melanoom naar het eerste lymfeklierstation is gemetastaseerd (stadium III melanoom) is het van belang het melanoom en de lymfekliermetastasen chirurgisch te verwijderen, omdat dit een goede kans op genezing biedt. Helaas zal bij ongeveer de helft van de patiënten met een volledig verwijderd stadium III melanoom, de ziekte zich op een later moment opnieuw openbaren (recidiveren). Dat komt doordat op het moment van operatie toch al een of enkele melanoomcellen zich verder verspreidden, maar op dat moment wel te klein waren om zichtbaar te zijn op een scan. Deze cellen kunnen later alsnog uitgroeien tot een zichtbare metastase. De kans op recidief is afhankelijk van de kenmerken van het oorspronkelijke melanoom en de lymfekliermetastasen op basis waarvan het stadium III melanoom ingedeeld wordt in stadium IIIA, IIIB en IIIC met een kans op overleving na 5 jaar van respectievelijk ongeveer 80, 60 en 40 procent. Dit wijst op een hoog risico op recidief, daarom is veel onderzoek gedaan naar de preventieve toepassing van een behandeling, aansluitend aan de chirurgische verwijdering (adjuvante behandeling). Sinds eind 2018 is adjuvante behandeling met de immune checkpoint inhibitoren nivolumab of pembrolizumab geregistreerd, omdat ze toen beide in een groot onderzoek aantoonden na een jaar tot minder recidieven te leiden. Tevens is de combinatie van een BRAF en MEK-remmer geregistreerd als adjuvante behandeling. Met deze behandeling is de kans op recidief kleiner en daarnaast is al aangetoond dat het tot een betere levensverwachting leidt. Naast deze doorbraken, wordt er ook veel onderzoek gedaan naar de adjuvante toepassing van DC-vaccinatie.

Dendritische celvaccinatie

DC-vaccinatie is een vorm van immuuntherapie die het immuunsysteem specifiek stimuleert om te reageren tegen tumorcellen. Bij DC-vaccinatie worden dendritische cellen of voorlopercellen daarvan uit de bloedbaan van de patiënt gehaald met een bloedfiltering (aferese). Hierna worden de cellen in de cleanroom van een laboratorium bewerkt. Voorlopercellen moeten eerst gedurende ruim een week gekweekt worden tot DC's. DC's worden vervolgens gematureerd en beladen met de juiste tumorantigenen. Deze gematureerde en beladen DC's worden teruggegeven aan de patiënt door middel van een injectie, zodat ze vervolgens in de lymfeklier de juiste T-cellen kunnen activeren. De T-cellen zullen dan de tumorantigenen op de melanoomcellen herkennen, de tumor binnendringen en deze vernietigen. De stappen van herkenning van de tumor door de DC en de maturatie van de DC worden op deze manier bij DC-vaccinatie overgenomen en de vaccinatie ondersteunt daardoor de tumor-immuniteitscyclus. Tot enkele jaren geleden werden altijd voorlopercellen

van DC's gebruikt, omdat deze in groteren getale voorkomen in het bloed dan de DC's. Sinds enkele jaren is het ook mogelijk de schaarse, van nature in het bloed circulerende DC's rechtstreeks te selecteren. Op deze manier is de langdurige kweekperiode met toevoeging van factoren die de effectiviteit van de DC's negatief kunnen beïnvloeden, niet langer nodig. Van nature in het bloed circulerende DC's zijn in twee grote groepen in te delen: myeloïde en plasmacytoïde DC's. Gebruik van zowel myeloïde als plasmacytoïde DC's in DC-vaccinaties toonden eerder in klinische studies aan dat ze tumorspecifieke T-cellen op kunnen wekken bij patiënten met een gemetastaseerd melanoom.

Onderzoek naar dendritische celvaccinatie bij melanoom

Er zijn meerdere klinische studies gedaan naar DC-vaccinatie bij melanoompatiënten die lieten zien dat deze experimentele behandeling tumorgerichte T-cellen kan opwekken. DC-vaccinatie heeft ook aangetoond slechts milde bijwerkingen als vermoeidheid, griepachtige verschijnselen of een ontstekingsreactie op de injectieplaats te hebben. Het uiteindelijke doel van de behandeling is afname van tumorgroei en dit wordt tot nu toe slechts bij een minderheid van de patiënten met een gemetastaseerd melanoom gezien. Dat komt waarschijnlijk doordat tumoren proberen te ontsnappen aan het immuunsysteem. Begrip van de mechanismen die tumoren hiervoor gebruiken is van belang, omdat dan ook gezocht kan worden naar mogelijkheden om deze te blokkeren. Bijvoorbeeld het combineren van DC-vaccinatie om tumorgerichte T-cellen op te wekken in combinatie met een therapie die de cellen die immuunreactie onderdrukken afremt. Wanneer tumoren nog niet ver ontwikkeld zijn, bijvoorbeeld als er geen zichtbare metastasen zijn op een scan, is de onderdrukking van het immuunsysteem door tumorcellen nog niet zo groot. Toepassing van immuuntherapie in een eerder stadium van de behandeling, zoals een adjuvante toepassing bij het stadium III melanoom, kan dus effectiever zijn dan toepassing bij een gemetastaseerd melanoom. Eerder onderzoek heeft ook laten zien dat stadium III melanoompatiënten die adjuvant behandeld werden met DC-vaccinatie, meer tumorspecifieke T-cellen ontwikkelden dan patiënten met een gemetastaseerd melanoom die behandeld werden met DC-vaccinatie. Daarnaast is achteraf de overleving van 78 stadium III melanoompatiënten die in het Radboudumc zijn behandeld met adjuvante DC-vaccinatie bekeken en vergeleken met eenzelfde groep patiënten uit het Erasmus MC die geen adjuvante therapie kregen. Uit deze data bleek dat patiënten na adjuvante DC-vaccinatie een langere overleving hadden. Een gegeven waaraan geen conclusies mogen worden verbonden, omdat bij het achteraf verzamelen van gegevens vertekening kan optreden die niet helemaal uit te sluiten is. Bijvoorbeeld de inclusie van studiepatiënten uit Nijmegen in vergelijking met inclusie van patiënten die niet aan studies hebben meegedaan uit het Erasmus MC, waardoor onbewust toch verschillen tussen beide groepen aanwezig kunnen zijn. Wel heeft de gevonden langere overleving de rationale voor onderzoek van adjuvante DC-vaccinatie bij het stadium III melanoom versterkt.

Het voordeel van DC-vaccinatie ten opzichte van immune checkpoint inhibitoren, is dat DC-vaccinatie met milde bijwerkingen gepaard gaat. Immune checkpoint inhibitoren kunnen ernstige bijwerkingen geven en enkele patiënten zullen overlijden als gevolg van de bijwerkingen. Waarschijnlijk komt dit door de tumorgerichte manier waarop DC-vaccinatie het immuunsysteem stimuleert, in tegenstelling tot een meer algemene stimulatie door immune checkpoint inhibitoren. Verdere optimalisatie van DC-vaccinatie en een juiste positionering in het huidige therapeutische landschap is de focus van huidig en toekomstig onderzoek.

Bevindingen van dit proefschrift

In dit proefschrift worden enkele manieren onderzocht waarop de toepassing en positie van DC-vaccinatie verder verbeterd kan worden: als onderdeel van combinatietherapie en het gebruik van natuurlijk circulerende DC's als adjuvante therapie. In eerder onderzoek is aangetoond dat cisplatin, een vorm van chemotherapie, verschillende effecten heeft op de anti-tumor immuunrespons. Cisplatinum kan bijvoorbeeld het aantal cellen die het immuunsysteem onderdrukt verminderen en expressie van immuunsuppressieve moleculen verlagen. In **hoofdstuk 2** worden de resultaten beschreven van een studie waarin 54 patiënten zijn behandeld met DC-vaccinatie. De helft van deze patiënten werd daarnaast behandeld met cisplatin. Aangetoond is dat het combineren van DC-vaccinatie met cisplatin mogelijk is, dat het veilig is en dat de combinatietherapie in staat is om tumorspecifieke T-cellen op te wekken. In de groep die beide middelen kreeg, werd geen verbetering van de immuunreactie aangetoond ten opzichte van de groep die alleen met DC-vaccinatie werd behandeld. Onze data suggereren niet dat het toevoegen van cisplatin de effectiviteit van DC-vaccinatie vergroot. Dit kan verschillende redenen hebben, waaronder het gebruikte interval tussen de vaccinatie en toediening van cisplatin of te weinig immunologisch effect van cisplatin in het gebruikte schema.

Na het afronden van de hierboven beschreven studie zijn immune checkpoint inhibitoren geregistreerd als behandeling. Doordat deze immune checkpoint inhibitoren op een andere plaats in de tumor-immuniteitscyclus ingrijpen dan DC-vaccinatie, kunnen immune checkpoint inhibitoren en DC-vaccinatie elkaar goed aanvullen. Bij het combineren van verschillende therapieën, is het belangrijk dat er niet te veel ernstige bijwerkingen optreden. Een groot voordeel van DC-vaccinatie is dat deze therapie slechts milde bijwerkingen heeft. Dat maakt het een ideale kandidaat voor combinatietherapie. Klinisch onderzoek naar de combinatie van DC-vaccinatie met PD-1-remmers is een belangrijke focus voor verder onderzoek.

Adjuvante behandeling van stadium III melanoom met DC-vaccinatie heeft waarschijnlijk meer effect dan behandeling van een gemetastaseerd melanoom omdat er minder tumorweefsel is en daardoor minder signaalstoffen, moleculen en cellen zijn die het immuunsysteem onderdrukken. In de studie beschreven in **hoofdstuk 3** zijn vijftien stadium III melanoompatiënten geïncludeerd waarbij zowel het melanoom als de lymfekliermetastasen volledig verwijderd zijn, waarna ze adjuvant behandeld zijn met DC-vaccinatie. In deze studie

is gebruik gemaakt van natuurlijk circulerende DC's. De gedachte was dat de combinatie van myeloïde met plasmacytoïde DC's in één vaccin waarschijnlijk effectiever is dan enkel één van beide soorten DC's. In deze studie zijn patiënten in drie groepen ingedeeld die elk met een ander type DC zijn behandeld: myeloïde DC's, plasmacytoïde DC's of een combinatie van myeloïde en plasmacytoïde DC's. Met dit onderzoek is de haalbaarheid en veiligheid van de toediening van de combinatie van beide cellen bevestigd. De combinatiebehandeling was tevens in staat tumorspecifieke en functionele T-cellen op te wekken bij 80 procent van de patiënten.

Van de patiënten in deze studie is ook de kwaliteit van leven onderzocht. De gemiddelde leeftijd van melanoompatiënten is zestig jaar, dat betekent ook dat veel patiënten op moment van diagnose betaald werk hebben. Daarnaast wordt het deel van de patiënten dat nooit een recidief van het melanoom zou krijgen onnodig blootgesteld aan de behandeling. Een behandeling die de kans op genezing verbetert én gepaard gaat met weinig bijwerkingen is altijd wenselijk. In de adjuvante setting bij relatief jonge patiënten die graag aan het werk blijven tijdens de behandeling is dat nog extra van belang. Het onderzoek beschreven in **hoofdstuk 4** laat zien dat de kwaliteit van leven van de patiënten tijdens adjuvante DC-vaccinatie verbetert. Dit is waarschijnlijk het effect van het herstel van de lymfeklieroperatie binnen twaalf weken voor het begin van de adjuvante therapie. Bij adjuvante behandelingen met PD-1-remmers en BRAF/MEK-remmers, sinds eind 2018 de standaardtherapie, wordt geen verbetering van de kwaliteit van leven gezien. Echter, hoewel de kwaliteit van leven belangrijk is, is het effect op de terugkeer van het melanoom doorslaggevender in de keuze voor een bepaalde soort therapie. Van adjuvante DC-vaccinatie wordt het klinische effect onderzocht in een groot fase III onderzoek waarbij in totaal ongeveer 150 patiënten worden behandeld met adjuvante DC-vaccinatie of een nepmedicijn (placebo). De resultaten hiervan volgen begin 2021.

Patiënten met een stadium III melanoom hebben een hoge kans op recidief. Er worden niet regelmatig scans verricht, omdat deze niet bewezen leiden tot een betere levensverwachting. Ook waren er geen data beschikbaar over recidieven binnen drie maanden na de lymfeklieroperatie, voor het begin van adjuvante therapie. In **hoofdstuk 5** worden de resultaten beschreven van de uitkomsten van de CT-scan die gemaakt is voordat patiënten konden beginnen met adjuvante DC-vaccinatie in het hierboven genoemde fase III onderzoek. Bij deze stadium IIIB of IIIC melanoompatiënten waren zowel het melanoom als de lymfekliermetastasen op dat moment al chirurgisch verwijderd. Voorafgaand aan de lymfeklieroperatie werden deze patiënten gescand, meestal met een PET-scan, om te verifiëren dat er behalve in de regionale lymfeklieren geen metastasen zijn. De patiënten kregen binnen twaalf weken na de lymfeklieroperatie opnieuw een scan, ditmaal een CT-scan. Hierop zagen we dat bij ongeveer één op de vijf patiënten op dat moment al terugkeer van de ziekte en bij de meerderheid was dit asymptomatisch. Dit is van belang omdat het tot een andere therapiekeuze kan leiden. Bij negen patiënten zijn deze metastasen bijvoorbeeld operatief verwijderd. Ook kan het de keuze van de soort adjuvante therapie beïnvloeden.

Daarnaast is het voor de evaluatiescan na drie maanden adjuvante behandeling belangrijk om een goede uitgangswaarde te hebben, zodat een metastase die al voor het begin van de behandeling aanwezig was, niet als tumorgroei wordt beschouwd en de therapie niet onterecht wordt gestaakt. Omdat sinds eind 2018 zowel immune checkpoint inhibitoren als doelgerichte therapie zijn geregistreerd voor de adjuvante behandeling van een stadium III melanoom, is deze informatie van belang voor de huidige behandeling van deze groep patiënten. Ons advies is dan ook om voor het begin van adjuvante behandeling eerst een CT-scan te verrichten.

Concluderend

De behandeling van het melanoom nam het afgelopen decennium een enorme vlucht. Ondanks de vele behaalde successen, komt ook tegenwoordig de meerderheid van de patiënten met een gemetastaseerd melanoom helaas uiteindelijk hieraan te overlijden. Een combinatie van verschillende therapieën vergroot mogelijk de effectiviteit van de behandeling, hoewel toename van bijwerkingen dan goed in het oog moet worden gehouden. Door het milde bijwerkingenprofiel is DC-vaccinatie een geschikte kandidaat als onderdeel van experimentele combinatietherapie met bijvoorbeeld PD-1-remmers.

De huidige adjuvante behandeling van het stadium III melanoom kan gepaard gaan met ernstige bijwerkingen. DC-vaccinatie heeft veel mildere bijwerkingen en leidt mogelijk ook tot een betere kwaliteit van leven dan de huidige adjuvante standaardbehandeling. Onderzoek naar de klinische effectiviteit van adjuvante DC-vaccinatie is daarom belangrijk. De eerste resultaten van de fase III studie die dit onderzoekt, worden begin 2021 verwacht.

In het huidige therapeutische landschap met adjuvante therapeutische opties is het belangrijk een scan te herhalen voor het begin van deze therapie. Een PET-scan voorafgaand aan de lymfeklieroperatie sluit het ontstaan van metastasen tussen deze operatie en het begin van adjuvante therapie onvoldoende uit.

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Curriculum Vitae

Martine Bloemendal werd geboren op 1 januari 1987 te Nijmegen. Nadat ze haar middelbare school aan het Dominicus College te Nijmegen in 2005 heeft afgerond, is ze gestart met de studie geneeskunde aan de Universiteit Antwerpen. In 2012 heeft ze met grote onderscheiding haar Master Geneeskunde aan diezelfde universiteit bepaald. In 2012 is ze bij de interne geneeskunde van het Máxima Medisch Centrum te Veldhoven gestart als arts-assistent niet in opleiding. In 2013 werd ze aangenomen voor de opleiding tot internist (opleider prof. dr. H.R. Haak en later dr. A.G. Lieverse, hoofdopleider prof. dr. R.P. Koopmans). In april 2016 heeft ze haar opleiding tijdelijk onderbroken om promotieonderzoek te doen bij de afdelingen Medische Oncologie en Tumor Immunologie van het Radboudumc te Nijmegen (promotoren prof. dr. W.R. Gerritsen en prof. dr. I.J.M. de Vries, copromotoren dr. K.F. Bol en dr. G. Schreibelt). Ze heeft tijdens haar onderzoek poliklinische zorg gedragen voor de melanoompatiënten die deelnamen aan de fase II studies en het fase III onderzoek die tijdens deze periode naar dit onderwerp liepen. Het resultaat van het promotietraject staat beschreven in dit proefschrift. In 2019 heeft ze haar opleiding tot internist hervat aan het Radboudumc te Nijmegen (hoofdopleider dr. G.M.M. Vervoort en voormalig hoofdopleider prof. dr. J. de Graaf). Aansluitend zal ze daar haar differentiatie tot medisch oncoloog starten (opleider dr. I.M.E. Desar).

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*Shared authorship